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MALIGNANT DISEASE IN THE GOLD COAST

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LITTLE is known about the types and incidence of tumours seen in the Gold Coast. MacFie (1922) considered that tumours were probably as common in the Gold Coast as elsewhere, sarcomas being more common than carcinomas. Findlay (1950) noted that primary liver carcinoma was common in West African troops, a finding confirmed in the Gold Coast by Edington (1956).

In an attempt to define the types and possibly the incidence of malignant tumours seen in the Gold Coast the autopsy protocols of the years 1923–55 and the records of biopsy specimens received during the years 1942–55 in the Medical Research Institute, Accra, have been scrutinised and the types of malignant tumour seen recorded in age and sex groups. The Institute is the only laboratory in the country where histological diagnosis is available and the material contained in the records should provide, with some reservations, a fair cross-section of the pathology of tumours occurring in the Gold Coast. In this connection it must be remembered that the facilities for the treatment of malignant disease are limited in many parts of the country and medical officers are therefore most likely to transmit specimens for diagnosis from conditions which they consider they can successfully treat. Consequently enlarged lymphatic glands and tumours of the skin or extremities are more likely to be considered worthy of investigation than malignant conditions affecting the internal organs. This is exemplified by the differing incidence of tumours in the autopsy and biopsy material.

It is felt that the disadvantage inherent in considering the biopsy material should be to some extent offset by the survey of the post-mortem records and that the investigation should indicate at least the common types of tumour seen and perhaps allow tentative conclusions to be drawn regarding incidence.

Where possible the histological diagnosis made by other pathologists has been confirmed by the author but little material is available prior to 1949 and the biopsy records prior to 1951 are in many instances incomplete with regard to age, sex and site of the lesion. This accounts for the large number of malignant tumours in the unclassified columns.

Even considering these drawbacks, however, in the absence of reliable vital statistics, it is thought that the findings arrived at in this communication are the most reliable which can be obtained at present and should form a basis for further work on the problems outlined.

The classification of tumours adopted is that of Willis (1953) and Table I illustrates the incidence and types of malignant disease seen in 4395 autopsies

TABLE I.—*Malignant Disease as a Cause of Death in 4395 Post Mortems Shown in Age and Sex Grouping*

	Under 1 year	1–5 years	6–15 years	16–25 years		26–35 years		36–45 years		46–55 years		Over 55 years		Total
				M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	
Total	593	395	245	485	298	794	276	622	114	229	43	236	56	4395
Group I—														
Carcinoma	—	1	2	3	3	8	—	16	4	9	3	9	3	61
Hepatoma	—	—	—	3	1	13	1	27	3	6	1	4	1	60
Group II—														
Sarcoma	—	—	2	2	—	3	—	3	—	—	—	1	—	11
Group III—														
Tumours of haemopoietic tissues	1	1	11	5	3	7	1	4	3	3	3	3	—	45
Group IV—														
Neural	—	—	2	—	—	—	—	—	—	—	—	—	—	2
Group V—														
Sundry special tumours	1	3	3	—	2	—	1	2	—	—	—	1	—	13
Unclassified	2	—	—	—	—	1	1	2	—	—	—	1	—	7
Total	4	5	20	13	9	32	4	54	10	18	7	19	4	199

In the unclassified tumours were meningioma (2), pituitary adenoma (1), neurinoma of intestine (1), congenital cyst of the liver (1) and a cavernous haemangioma of the brain (1).

in age and sex groups. Deaths from unnatural causes have been excluded from the final autopsy total. Excluding the 6 simple tumours, 193 patients had died of malignant disease (4·4 per cent). When it is considered that many of the post-mortems were performed at the request of the Coroner on subjects who had died suddenly and when the small number of autopsies in the older age groups is considered it can be stated that there is no scarcity of malignant disease in the Gold Coast. Malignant disease was most common in the 6–15-year and over 35-year-old age groups.

During the years 1942–55 1000 biopsies from malignant tumours had been received in the Institute. The tumours seen in the autopsy and biopsy material are considered together in their respective groups throughout this paper and the total seen, in accordance with the classification of Willis (1953) is shown in Table II.

Group I

Tumours of epithelial tissues

Carcinoma was the most common type of malignant tumour seen (64 per cent) and the site and sex incidence are shown in Table III. In 75 of the 766 tumours (9·8 per cent) the site could not be specified.

Carcinoma of the skin

The most common type of malignant tumour seen in the Gold Coast is the squamous celled carcinoma of the skin (166 excluding cervical carcinoma). Basal celled carcinoma would appear to be relatively rare, only 12 specimens being noted. The remaining tumours were adnexal tumours of the skin of doubtful malignancy.

TABLE II.—*The Types of Malignant Tumours Seen in* 4395 *Autopsies and* 1,000 *Biopsies*

Classification	4395 autopsies		1000 biopsies			
	Male	Female	Male	Female	No details	Totals
I.—Tumours of epithelial tissues	98	23	169	211	265	766
II.—Tumours of non-haemopoietic mesenchymal tissues	10	1	25	31	102	169
III.—Tumours of haemopoietic tissues	31	14	28	5	51	129
IV.—Tumours of neural tissue	2	—	7	8	10	27
V.—Sundry special tumours	8	5	14	39	35	101
Total	149	43	243	294	463	1192*

* There was one unclassified malignant tumour in the autopsy material bringing the total to 1193.

TABLE III.—*Site and Sex Incidence of* 766 *Carcinomas Seen in* 1193 *Malignant Tumours*

Site of tumour	4395 autopsies		1000 biopsies			
	Male	Female	Male	Female	No details	Total
Skin	1	—	60	42	83	186
Liver	53	7	14	2	15	91
Unclassified	1	—	—	—	74	75
Uterus and cervix	—	1	—	70	—	71
Breast	—	2	2	40	20	64
Stomach	7	5	16	9	6	43
Salivary glands	—	—	13	7	20	40
Ovary	—	4	—	29	—	33
Bladder	13	1	7	1	8	30
Prostate	3	—	22	—	—	25
Adamantinoma	—	—	10	4	8	22
Large intestine	2	—	7	2	9	20
Nasopharynx	1	—	1	2	10	14
Thyroid	—	—	5	1	7	13
Pancreas	8	—	2	—	1	11
Testis	—	—	10	—	—	10
Kidney	3	—	—	1	3	7
Lung	2	2	—	—	—	4
Small intestine	1	—	—	—	1	2
Larynx	1	1	—	—	—	2
Oesophagus	1	—	—	—	—	1
Gall bladder	1	—	—	—	—	1
Lachrymal gland	—	—	—	1	—	1
Total	98	23	169	211	265	766

Fifty-seven of the squamous celled carcinomas could not be classified and the sites of the remaining 109 tumours are shown in Table IV.

There appeared to be little sex differentiation in incidence. The ages ranged from 18–60 with a mean of 44 years. The youngest patient was a boy of 18 years with an epithelioma of the penis. Chronic ulceration would appear to be a predisposing factor. The low incidence of basal celled carcinoma is probably related to the short life expectancy of the Gold Coast African.

TABLE IV.—*Sites of 109 Squamous Celled Carcinomas*

Site	Number	Site	Number
Lower leg and foot	24	Hand and forearm	6
Orbital area	16	Face	5
Penis	16	Neck	4
Mouth and tongue	13	Arms	3
Vulva	10	Others	6
Scrotum	6		

Carcinoma of the liver

Apart from the skin the liver would appear to be the organ most frequently affected by malignant disease (7·6 per cent of all malignant tumours)' In the autopsy material the incidence of carcinoma of the liver was 2·2 per cent in adult males and 0·9 per cent in adult females. The incidence in age and sex groups is shown in Table I. In the biopsy material the ages ranged from 12–65 with a mean of 35 years. Thirty-eight sections were available for study. Primary liver celled carcinomas arising in cirrhotic livers were noted in 34 instances and without accompanying cirrhosis in one instance. The remaining 3 tumours were cholangiocellular carcinomas. These findings agree closely with those of Berman's (1951) in the Bantu. The aetiology of cirrhosis and cancer of the liver in the Gold Coast has been discussed in a previous communication (Edington, 1956). Primary liver celled carcinoma almost invariably arises in a cirrhotic liver and malnutrition is generally considered an important aetiological agent in the production of cirrhosis in the tropics. In the Gold Coast the incidence of Kwashiorkor and stellate fibrosis show an equal sex incidence in children. Thus if the sex incidence of carcinoma of the liver is correct in the autopsy material (the incidence of cirrhosis was 15 per cent in males and 3 per cent in females) it would suggest that malnutrition in childhood is not the sole agent responsible for cirrhosis and carcinoma of the liver in the adult in the Gold Coast.

Carcinoma of the uterus and cervix

There were 16 carcinomas of the body of the uterus (ages 27–62 with a mean of 43 years) and 55 of the cervix (ages 26–65 with a mean of 47 years). The age incidence of cervical carcinoma is similar to that recorded in Europe whereas the age incidence of uterine carcinoma is considerably lower. In no instance was concomitant schistosomiasis noted.

Carcinoma of the breast and prostate

Eighty-two biopsy specimens of the breast had been received. Of these 20 showed fibroadenosis or cystic hyperplasia (one male included). Of the 64 cancers (including 2 in the autopsy material) the sex was noted in 44 (two males) and the ages ranged from 20–72 with a mean of 45 years. Breast cancer in males would appear to be relatively more common in the Gold Coast than in Europe the aetiological agent in its production perhaps being gynaecomastia, a not uncommon finding in malnutrition and cirrhosis of the liver. The relatively few biopsies showing fibroadenosis or cystic hyperplasia is in contrast with the findings in prostatic pathology where fibroadenomatous hyperplasia was diagnosed in 62 specimens received and malignancy in 22. The findings would suggest that fibroadenosis and cystic hyperplasia of the breast are less common in the Gold Coast than in Europe but clinical confirmation of this conclusion is required.

Carcinoma of the Stomach

The relatively large number of carcinomas of the stomach seen was surprising (3·6 per cent of malignant tumours). In the 2366 autopsies performed on adult males there were 7 carcinomas of the stomach (0·3 per cent). The comparable figures for females being 787 and 5 (0·6 per cent). It has generally been considered that peptic ulceration is rare in the Gold Coast African and specimens are rarely received for histological diagnosis—only 4 were received in the years 1942–55. The post-mortem records were therefore searched to discover the incidence of perforated peptic ulcers. In the 4395 post mortems discussed in this paper death was due to a perforated peptic ulcer in 21 instances. There were 12 perforated duodenal ulcers (all males aged 29–67 with a mean of 45 years), four perforated prepyloric ulcers (all males aged 45–70 with a mean of 53 years) and 5 perforated gastric ulcers (2 in females, ages not available). Duodenal ulcer therefore occurs in the male African in the Gold Coast and gastric ulcer is probably less common and occurs in both sexes. The occupations of those dying of a perforated duodenal ulcer were varied but included Northern Territory labourers and farmers showing that urbanisation was not the underlying factor. There was no sex difference in the incidence of gastric carcinoma, the ages varying from 25–66 with a mean of 46 years. Rose (1955) suggested that schistosomiasis might be a causative agent in peptic ulceration in Nigeria but the ova have not been noted either in gastric carcinoma or peptic ulceration in the Gold Coast.

Carcinoma of the salivary glands

There were 40 specimens of salivary gland tumours and one lachrymal gland tumour exhibiting the histopathology of a mixed salivary tumour. There were 2 simple adenomas, one unclassified tumour in a male aged 52 years and 3 adenocarcinomas. The remaining 34 specimens exhibited the pathology of the mixed salivary tumour. Three of these were considered more than locally malignant as metastases in lymph glands had occurred. In only one instance were the sections from these 3 tumours available for study and the histopathology of the tumour did not appear to differ from that seen in the other 27 sections of mixed salivary tumours available. All histological patterns were seen from the cribriform to the mucinous with a few epithelial strands and areas of "cartilage" present. Bone was present in one tumour. The majority of the tumours arose from the parotid glands but 6 of the tumours were situated in the submaxillary glands and one each in the upper lip, palate and cheek. The sex was given in 20 instances there being 13 males (ages varying from 11–52 years with a mean of 33) and 7 females (ages varying from 12–34 years with a mean of 27).

Carcinoma of the ovary

Simple ovarian cysts (follicular, serous and pseudomucinous cystadenomas) and dermoids are not uncommon. Thirty-three ovarian tumours were considered to be malignant. The ages ranged from 6–54 with a mean of 24 years. Blocks from 22 of these tumours were available for study and the most common type of tumour was the dysgerminoma (10). In 2 instances these tumours were bilateral and 2 girls of 6 years were affected. The differentiation from an anaplastic round

celled carcinoma was difficult in a number of these cases but the tumours showed a uniform type of cellular pathology and a number of pathologists were consulted before a diagnosis was reached. Davies and Wilson (1954) noted bilateral ovarian tumours in 2 young girls which histologically proved to be undifferentiated malignant round cell tumours of uncertain nature and three of the tumours in this series diagnosed as dysgerminoma would fit this description. Adenocarcinoma (5) affected the older age groups. Carcinoma simplex (4) and granulosa cell tumours (3) were the remaining types of tumours seen. In addition 3 solid teratomas are described in Group V. Bilharzia ova have not been implicated in the pathology of the ovary in the Gold Coast apart from a cyst seen in a girl of 16 years containing an adult schistosome.

Carcinoma of the bladder and large intestine

Thirty cancers of the bladder were seen. It was more common in males (10 : 1) The ages ranged from 19–65 years with a mean of 37. Schistome ova were noted in about 30 per cent of the biopsy and 71 per cent of the autopsy material. No specialised investigations have however been performed so these figures are undoubtedly low. The youngest patient (19 years) had an extensive and severe schistosome infestation and the clinical findings have been described by Gamble (1954). The importance of bilharzia infestation in the aetiology of carcinoma of the bladder is controversial. Davies and Wilson (1954) noted a low incidence of schistosomiasis in Kampala but the age distribution of cancer of the bladder was higher than that recorded in the Gold Coast. Gelfand (1950) did not consider that bilharzial disease was a cause of bladder cancer in South Central Africa, the autopsy incidence in the Bantu being 0·25 per cent, a similar figure to the incidence in autopsies in the Gold Coast. In the Gold Coast however the age distribution of the autopsy material must be considered and these figures cannot be compared with records from Europe owing to the small number of autopsies in the older age groups. Willis, (1953) stated that the age distribution peak of epithelial tumours of the urinary passages was early in the seventh decade and the mean age of death in his 37 cases of carcinoma of the bladder was 64 years. The difference in sex incidence may be an argument against schistosomiasis being a factor in the production of bladder cancer. In addition, if bilharzia infestation were the cause of bladder carcinoma it could be argued that the incidence of rectal carcinoma should be high. In this material there were 20 tumours of the large intestine, 12 being adenocarcinomas of the rectum. In only 6 instances was the sex given (4 males and 2 females) and the ages ranged from 25–59 with a mean of 41 years. In no instance were bilharzia ova noted although they have been seen in two benign rectal papillomata. It should be remembered however that *S. mansoni* is less common in the Gold Coast than *S. haematobium* and heavy infections of *S. mansoni* in contradistinction to *S. haematobium* have not been recorded. In spite of the arguments against schistosomiasis being responsible for bladder carcinoma the present writer considers that it cannot definitely be excluded as an aetiological agent with the information existing at present.

Adamantinoma

Twenty-two adamantinomas of the jaw were seen. The sex was given in 14, there being 10 males (ages varying from 12–53 with a mean of 32 years) and 4

females (ages ranging from 15–50 with a mean of 35 years). The age incidence agrees with that described by Willis (1953) and no conclusions can be drawn from the sex incidence.

Carcinoma of the thyroid gland

Eighty-three specimens of thyroid tissue had been submitted for histological diagnosis. Carcinomatous change was considered to have occurred in 13 instances (11 were papillary adenocarcinoma and 2 specimens were aberrant thyroid tissue removed from the lateral aspect of the neck and reported as malignant.) In addition a solid teratoma and a dermoid cyst were seen. Apart from 2 foetal adenomata the remaining 66 specimens showed the histopathology of the colloid type of adenomatous goitre. Degenerative and cystic changes were common but there was a noticeable absence of lymphocytic infiltration. Neither Riedel's struma nor Hashimoto's disease were seen. The majority of the patients were from the Northern Territories. Toxic symptoms were rarely recorded. The age and sex incidence was available in 46 biopsies and is shown in Table V.

TABLE V.—*Age and Sex Incidence in Thyroid Disease*

	Simple goitre			Carcinoma		
Sex	Number	Age range	Mean age	Number	Age range	Mean age
Females . .	33	16–50	36	5	30–52	43
Males . .	7	19–50	39	1	—	50

It is known that endemic goitre is common in the Navrongo and Bawku areas of the Northern Territories of the Gold Coast but there are no records of its occurrence in the Colony. Annual reports by various medical officers from these districts have emphasised its frequency in the out-patient departments and proposed iodisation of salt as a preventive measure. The aetiology of endemic goitre in South Africa (1955) and Sierra Leone (Wilson, *et al* 1954) has been discussed and is outside the present scope of this paper. It is probable, however, that the incidence of carcinoma of the thyroid will be found to be high in these areas in the Gold Coast and there is a fruitful field for research on this problem. No information is available on the incidence of cretinism or deaf mutism.

Carcinoma of the pancreas

Eleven were recorded. In the 10 instances where the sex was noted the tumours were all in males. The ages ranged from 18–60 with a mean of 41 years. In view of the fact that Kwashiorkor is common in the Gold Coast and that it primarily affects the pancreas it was thought that the incidence of carcinoma might be high. This would not appear to be so. Unfortunately there is no information available on the incidents of fibrosis of the pancreas and this condition was considered an aetiological agent in the cause of death in only one subject in the autopsy records, a male aged 30 years. Haemorrhagic pancreatitis has been recorded as a cause of death in 4 instances all in young adult males.

The calcification and fibrosis of the pancreas described by Zuidema (1955) in Indonesian patients and considered to be due to protein deficiency would not appear to be common in the Gold Coast. Fibrocystic disease of the pancreas

would also appear to be uncommon. No evidence has been produced to implicate nutritional disease in the aetiology of carcinoma of the pancreas.

The less common carcinomas

There was little of note in the nasopharyngeal tumours. There were 10 carcinomas of the testis, 3 were unclassified and the remainder were seminoma (5) interstitial cell tumour (1) and chorionepithelioma (1). In addition there were 2 solid teratomas and one sarcoma in a boy aged 17 years.

In the kidney there were 2 hypernephromas and 2 papillary adenocarcinomas. Wilm's embryoma was the most common type of tumour of the kidney and is considered in its appropriate group.

Cancer of the lung would appear to be rare. In the Gold Coast pipe smoking is generally considered to be more popular than cigarette smoking especially with older men and women, a number of whom are heavy smokers. Cigarette smoking is however, common and is increasing in amount but relatively few of the population would be classed as heavy smokers. Smog of course is not seen but, especially in the North, wood smoke is constantly inhaled and charcoal braziers are employed. Carbon monoxide poisoning may be a cause of unexplained deaths in huts in the cold season. Little more can be said on the lung cancer—smoking controversy but it will be interesting to note if the incidence of lung cancer increases in the future. Two simple lipomas of the pleura were seen in the biopsy material.

Cholecystitis and gallstones are rare in the Gold Coast (Edington, 1956). This is surprising when the high incidence of infectious diseases and haemolytic anaemias is remembered. Carcinoma of the gall bladder was also rare in the material reviewed as was carcinoma of the lachrymal gland.

Group II

Tumours of non-haemopoietic mesenchymal tissues

Considerable difficulty was experienced in classifying the tumours seen in this group. Sarcoma would appear to have been more common in the pre-1948 autopsy and biopsy material and consequently the tissues available for study were limited. In addition the details given in many biopsy specimens were meagre, clinical examination was not possible and follow up results virtually *nil*. In view of these deficiencies it was difficult in a number of instances to classify the tumours as benign or malignant. The tumours causing the most anxiety were the fibromas or fibromyxomas showing considerable cellular activity and in particular, certain of the angiomatous tumours (sclerosing haemangioma, Kaposi's sarcoma, angiosarcoma and the active highly vascularised fibrous tissue seen in certain chronic ulcers). The most common of the simple tumours seen in this group were fibroma (105) uterine fibroids (64), angioma (47), giant cell tumours of bone, tendon sheaths and gum (45). Neurofibromatosis was seen in 20 instances.

In the autopsy material a meningioma caused death in 2 instances and a cavernous angioma of the brain in one. The types of sarcoma seen are shown in Table VI. The high number unclassified has already been explained and the great majority of these from the description available were probably fibrosarcomas.

The sex was given in 32 of the 48 fibrosarcomas. There were 20 males (ages ranging from 4 months to 65 years, mean 27) and 12 females (ages ranging from

TABLE VI.—*Types of Sarcoma Seen in* 4395 *Autopsies and* 1000 *Malignant Biopsy Specimens*

Unclassified	56	Kaposis sarcoma	10
Fibrosarcoma	48	Chondrosarcoma	5
Angiosarcoma	24	Rhabdomyosarcoma	5
Osteogenic sarcoma	16	Sarcoma botryoides	5
Total	169		

7 months to 53 years, mean 35). The most commonly recorded sites were thigh (8), uterus (5), desmoid tumour of the abdominal muscles (4), fibromyxomatous type of tumour in the retroperitoneal tissues (4), groin (4) and buttocks (3). It is of interest to note the occurrence of a sarcoma of the breast (1), heart (1), and stomach (1).

The most common site of the osteogenic sarcomas was in the jaw region and usually occurred in children aged about 7 years.

Osteoclastomas (45) have been excluded from the list of malignant tumours seen as it was found impossible to segregate accurately these tumours from the benign tumours of tendon sheaths and giant cell epulides on histological examination alone in the absence of clinical and radiological information. Osteoclastomas, however, would appear to be more common in the Gold Coast than in England (Eyre-Brook, 1956). These tumours were predominantly found in women in the third decade (sex ratio 6 : 1).

The angiosarcomas and Kaposi's tumours were most commonly sited on the lower limbs. In one autopsy the cause of death was given as Kaposi's sarcomatosis with multiple lesions in the small intestine. As has already been pointed out the histopathological classification of these tumours in the absence of careful clinical studies is difficult and the types of angiomatous tumours seen in the Gold Coast African should be the subject of special investigation in the future.

Group III

Tumours of haemopoietic tissues

The types of malignant disease seen are shown in Table VII.

TABLE VII.—*Tumours of the Haemopoietic Tissues Seen in* 4395 *Autopsies and* 1000 *Biopsy Specimens*

Type of tumour	Biopsy specimens	Autopsies	Total
Lymphosarcoma and lymphatic leukaemia	28	17	45
Hodgkin's disease	18	12	30
Reticulosarcoma	17	5	22
Unclassified	11	2	13
Myelomatosis	6	1	7
Leukaemia, myeloid	2	3	5
unclassified	—	5	5
monocytic	2	—	2
Total	84	45	129

In the 13 unclassified tumours were two cases of histiocytic medullary reticulosis, one of Letterer Siwe's disease and one of mycosis fungoides which has already been described (Ashworth and Edington, 1954).

Lymphosarcoma was seen in 37 instances and the sex was noted in 23, there being 19 males (ages ranging from 4–59 with a mean of 20 years) and 4 females (ages ranging from 6–30 with a mean of 12 years). The most common sites were the upper abdominal glands and the maxillary region, young children especially being affected. There were 8 cases of lymphatic leukaemia.

The sex was only noted in Hodgkin's disease in 13 instances there being 8 males and 5 females, the mean ages being 36 and 29 years respectively.

There was little of interest to note in the remaining tumours apart from two reticulosarcomas of the retroperitoneal tissues and one of the small intestine. Reticulosarcomas occurred in an older age group when compared with lymphosarcoma, the mean age in 8 males being 43 years. It is considered that the incidence of leukaemia is much too low as the diagnosis is usually made on haematological findings and consequently these cases would not be considered in this review.

Giant follicular lymphoma, polycythaemia and lipoid storage disease have yet to be diagnosed in the Gold Coast.

Group IV

Tumours of neural tissue

Malignant tumours of neural tissue with the exception of the retinoblastoma are rarely diagnosed in the Gold Coast. The types of tumour seen are shown in Table VIII.

TABLE VIII.—*Tumours of Neural Tissue*

Retinoblastoma	18	Chromaffinoma	1
Glioma	3	Cartoid body tumour	1
Neurileumenoma	3	Neuroblastoma	1

The sex in 13 of the retinoblastomas was females 7 (ages 1½–6 with a mean of 3 years, 4 months) and males 6 (ages 2–8 with a mean of 4½ years). One neuroblastoma (mediastinal) was seen in a newly-born male baby and one astrocytoma involving the third ventricle and frontal lobes of the brain was seen at autopsy in a boy aged 13 years.

Group V

Sundry special tumours

The types of malignant tumours seen are shown in Table IX.

TABLE IX.—*Sundry Malignant Tumours Seen in* 4395 *Autopsy and* 1000 *Biopsy Specimens*

Type	Autopsy	Biopsy	Total
Malignant melanoma	3	60	63
Chorionepthelioma	1	20	21
Teratoma	2	6	8
Nephroblastoma	5	2	7
Unclassified	2	—	2
Total	13	88	101

Seventy-four specimens of melanoma were seen. Of these only 11 were considered benign and the majority of these were small pigmented tumours of the

conjunctivae and were most commonly seen in children. This is understandable as patients with tumours of the eye readily present themselves for treatment and the tumours are noticed when small. Melanotic tumours of the skin were usually described as fungating and it is probable that patients with a small benign melanoma of the skin did not attend for medical treatment. The sex was noted in 33 of the malignant tumours there being 15 males (ages ranging from 45–72 with a mean of 55 years) and 18 females (ages ranging from 36–67 with a mean of 51 years). By far the most common site was the foot especially the plantar aspect and secondary involvement of the inguinal glands was not uncommon in the material. The sites recorded were foot (32), eye (6), abdominal wall (1) and nasal region (1). No sex difference was noted.

Chorionepithelioma was diagnosed in 21 instances. The ages ranged from 19–35 with a mean of 27 years. The majority of these tumours were diagnosed by uterine curettage, in many instances supplemented by a quantitative male toad pregnancy test. Hysterectomy confirmed the diagnosis on four occasions. In two instances the lesions presented in the vaginal wall and in one instance peritoneal secondaries followed a ruptured tubal pregnancy. Hydatidiform mole was not uncommon in the biopsy material, 33 specimens being received. The ages ranged from 19–40 years with a mean of 24. The ratio of hydatidiform mole to chorionepithelioma is much too low and this can probably be explained by the macroscopic diagnosis of a mole being made by medical officers in outstations without recourse to histological examination. Curettage, however, is necessary for the diagnosis of chorionepithelioma and explains its high ratio in the biopsy specimens. It can be stated that the incidence of hydratidiform mole and chorionepithelioma is high in the Gold Coast and the age incidence recorded agrees with that recorded by King (1956) in Chinese.

Eight solid teratoma were seen, the sites recorded being ovary (3), testis (2), thyroid (1) and mediastinum (2). Dermoid cysts were most commonly sited in the ovary (9), orbital region (3) and neck (2). One woman died of peritonitis following a ruptured dermoid cyst of the ovary due to trauma.

Nephroblastoma occurred in the younger age groups. The unclassified tumours in the autopsy material were two embryonic tumours, one situated in the mediastinum of a newly-born child.

DISCUSSION

The tumours seen in the Gold Coast have been discussed in their relevant groups in the classification adopted. It has been previously noted that the accuracy of the incidence of these tumours is debatable. In an attempt to assess the accuracy of the incidence of the various types of tumours seen the results have been compared with the incidence of malignant disease as seen in Uganda (Davies and Wilson, 1954), in Nigeria (Elmes and Baldwin, 1947) and French West Africa (Camain, 1954). The results are shown in Table X. It is doubtful, in view of the methods of investigation and classification employed by the various writers, if the total figures shown in Table X are 100 per cent comparable. In the Gold Coast, for example, giant cell tumours have been excluded from the total list of malignant tumours whereas in Nigeria they were included. On the other hand leukaemia was not considered in the Nigerian material. Various other discrepancies have been noted but it is not considered that gross inaccuracies have resulted.

TABLE X.—*The Incidence of Various Tumours Compared to the Total Incidence of Malignant Disease Seen in Africans in the Gold Coast, Uganda, Nigeria and French West Africa*

Percentage figures shown in brackets.

Type of tumours	Gold Coast 1193	Uganda 734	Nigeria 1000	French West Africa 1884
Carcinoma				
Squamous celled of skin	166 (13·9)	100 (13·6)	122 (12·2)	235 (12·5)
Basal celled of skin	12 (1·0)	1 (0·1)	?	24 (1·3)
Liver	91 (7·6)	56 (7·6)	81 (8·1)	484 (25·7)
Uterus and cervix	71 (6·0)	70 (9·5)	68 (6·8)	100 (5·3)
Breast	64 (5·4)	29 (4·0)	84 (8·4)	103 (5·4)
Stomach	43 (3·6)	23 (3·1)	22 (2·2)	43 (2·3)
Salivary glands	40 (3·3)	10 (1·4)	60 (6·0)	66 (3·5)
Ovary	33 (2·8)	36 (4·9)	27 (2·7)	36 (2·0)
Bladder	30 (2·5)	20 (2·7)	18 (1·8)	31 (1·6)
Prostate	25 (2·1)	19 (2·6)	8 (0·8)	24 (1·3)
Adamantinoma	22 (1·9)	10 (1·4)	18 (1·8)	??50 (2·7)
Large intestine	20 (1·7)	19 (2·6)	14 (1·4)	?? < 23
Nasopharynx	14 (1·2)	18 (2·5)	19 (1·9)	?
Thyroid	13 (1·1)	5 (0·8)	14 (1·4)	17 (0·9)
Pancreas	11 (0·9)	7 (1·0)	7 (0·7)	13 (0·7)
Testis	10 (0·8)	1 (0·1)	7 (0·7)	5 (0·3)
Kidney	7 (0·6)	5 (0·8)	10 (1·0)	12 (0·6)
Lung	4 (0·3)	8 (1·0)	5 (0·5)	5 (0·3)
Sarcoma	159 (13·3)	57 (7·8)	137 (13·7)	95 (5·0)
Kaposi's	10 (0·8)	27 (3·7)	24 (2·4)	5 (0·3)
Osteoclastoma	45 (3·6)†	?	23 (2·3)	?
Lymphosarcoma and lymphatic leukaemia	45 (3·8)	37 (5·0)	53 (5·3)	119 (6·3)
Hodgkins	30 (2·5)	7 (1·0)	—	35 (1·9)
Reticulosarcoma	22 (1·9)	6 (0·8)	—	63 (3·3)
Myeloma	7 (0·6)	2 (0·3)	—	12 (0·6)
Leukaemia	9 (0·8)	11 (1·5)	—	16 (0·8)
Retinoblastoma	18 (1·5)	3 (0·4)	21 (2·1)	13 (0·7)
Glioma	3 (0·3)	—	3 (0·3)	8 (0·4)
Melanoma	63 (5·3)	13 (1·8)	62 (6·2)	49 (2·6)
Chorionepithelioma	21 (1·8)	3 (0·4)	9 (0·9)	?
Teratoma	8 (0·7)	14 (1·9)*	3 (0·3)	?
Nephroblastoma	7 (0·6)	12 (1·6)	4 (0·4)	?

* Fourteen of kidney

† These giant cell tumours are included only to compare the incidence with that of Elmes and Baldwin (1947).

From Table X it will be seen that there is remarkably close agreement in the incidence of carcinoma in these four territories. The incidence of carcinoma of the liver was however much higher in French West Africa. Carcinoma of the larynx was rare excepting French West Africa (0·8 per cent) as was carcinoma of the oesophagus excepting Uganda (2·3 per cent). Carcinoma of the conjuctiva (31) was also more common in French West Africa but these tumours have included squamous celled carcinomas which were recorded as epitheliomas of the orbital area in the Gold Coast (16) and there were also 21 carcinomas of the orbital region recorded in Uganda.

The incidence of sarcomas in the various territories shows some variation and does not agree as closely as the incidence of carcinoma. The difficulty in histological diagnosis of many of these tumours has however been previously discussed and it is considered that the figures therefore, show surprisingly close agreement. It is also considered that this remark applies to tumours described in the remaining three groups shown in Table X. When the various methods utilised by the investigators are considered Table X would suggest that the overall pattern of malignant disease as described in this paper in the Gold Coast is reasonably accurate. It can thus be concluded that carcinoma is common in the Gold Coast and that the most common types met with are the squamous celled carcinoma of the skin, primary liver celled carcinoma, carcinoma of the uterus and breast and surprisingly, fifth in incidence, carcinoma of the stomach. No evidence has been produced to incriminate schistosomiasis as an aetiological agent in the production of carcinoma of the bladder.

Further investigations are required to determine accurately the types and incidence of sarcomas seen in the Gold Coast. Tumours of the haemopoietic tissues are common and should prove a fruitful field of research in the future. Malignant melanoma is also common, and as already stated, trauma would appear to be a precipitating factor in its aetiology. Protective footwear should decrease its incidence.

The figures given in most publications from Africa which consider malignant disease are small when compared to those recorded in Europe but gradually the pattern of malignant disease is emerging. In the four territories considered it shows reasonably close agreement. At present few conclusions can be drawn but it is doubtful if malnutrition plays a great part in the aetiology of malignant disease in tropical Africa. Trauma, chronic ulceration and reticuloendothelial hyperplasia due to multiple infestations and infections may well be the most important predisposing factors.

SUMMARY AND CONCLUSIONS

Autopsy protocols and biopsy records have been scrutinised in the Medical Research Institute, Accra, and all available material has been examined. The type of malignant disease seen has been recorded in age and sex groups. The results have been compared with the incidence of malignant disease in other parts of tropical Africa and it is concluded that the findings recorded in this paper portray reasonably accurately the incidence of the various malignant tumours which occur in the Gold Coast.

I am most grateful to Professor C. V. Harrison for his advice willingly given on many occasions during the last five years, to Messrs Laryea and Tamakloe for technical assistance and to Dr. E. Akwei, Chief Medical Officer, Gold Coast, for permission to publish.

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PRIMARY CARCINOMA OF THE LIVER IN AFRICA

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ALL reports reviewing the frequency of liver carcinoma in Africa, emphasise the high incidence of this neoplasm observed among the indigenous races (Ninard, 1950; Berman, 1951; Oettlé, 1956). From a study of liver cancer in the native townships around Johannesburg, it can be concluded that this increase is absolute and not due to a relative absence of other tumours (Oettlé and Higginson, 1956).

The present paper draws attention to the peculiar position occupied by the hepatocellular tumour in relation to this increase. The possible development and aetiological background of this type of growth is discussed in view of certain similarities to experimental animal hepatomas. Attention is also drawn to the view that despite its significance in experimental liver cancer, malnutrition is unlikely to be the only factor causing this high frequency in the African Negro.

MATERIAL AND METHODS

This study is based on 53 primary carcinomas of the liver observed at necropsy at Baragwanath Hospital, Johannesburgh. Fifty-two tumours were from Bantu Negro subjects and one from a patient of Eurafrican descent. Surgical or needle biopsies were available in 15 of these, also in a further 36 carcinomas. In addition to the routine histological stains, mitochondria were demonstrated in liver biopsy material fixed in neutral formalin by Mallory's phosphotungstic haematoxylin after oxidation with potassium permanganate.

To estimate the proportion of multinucleate cells, sections were cut at 4 μ and groups of approximately 200–300 cells in contiguous fields were counted at 1250 magnification. Only cells with nuclei were included.

GENERAL PATHOLOGICAL CONSIDERATIONS

The basic pathological picture of primary liver carcinoma would appear to be similar in all countries (Ninard, 1950; Berman, 1951; Edmondson and Steiner, 1954). Some pathological features of the tumours in the present series are illustrated in Fig. 1–5, and are identical to those previously described in this region (Berman, 1951). Certain characteristics, however, require amplification.

Significance of Tumour-cell Type

Classically, liver tumours have been divided into hepatocellular and cholangiocellular types, but the significance of this classification has not yet been established. Berman (1951) states that "while the classification of primary liver cancer into hepatocellular and cholangiocellular carcinoma is useful for descriptive purposes, this division cannot be taken to mean that because a tumour assumes a particular pattern it is necessarily derived from the tissue it simulates" (p. 96). Ninard

(1950), however, regards hepatocellular tumours as specific and characteristic of the liver, and considers the cholangiocellular cell type non-specific and similar to any tumour of the intra- or extra-hepatic biliary system. On histogenic grounds, tumours of the liver cells and cholangioles should be regarded as " hepatocellular " or " liver cell type ", and tumours of the intra-hepatic bile ducts " cholangiocellular " or " bile duct type " ; but, while it would appear that the liver cell and bile duct anlages develop early and remain distinct, some workers believe cells of one type may develop into cells of the other.

Additional terminological confusion has been caused by difficulty in distinguishing the adenoid hepatocellular tumour from the cholangiocellular tumour (Fig. 4), and the former has sometimes been regarded as of the bile duct type. When present, we have regarded bile formation as pathognomic of the liver cell type.

In Table I the proportion of tumours of each cell type in the present series is shown, and is of the same order as that reported by Berman (1951) in mine workers, and in West African natives by Payet *et al.* (1953). The proportion of cholangiocellular tumours is lower, however, than that observed in Central Africa (Davies, 1952) and much lower than reported from America by Edmondson and Steiner (1954), although the latter authors took into account the adenoid hepatocellular type.

EXPLANATION OF PLATES

FIG. 1.—Typical primary carcinoma in a liver showing a coarse nodular cirrhosis. Many of the parenchymal nodules in this specimen are undergoing malignant change. Zulu, male, aged 34 years.

FIG. 2.—Sections from same liver as Fig. 1 showing numerous foci of hepatocellular carcinoma. The surrounding liver shows foci of collapse and fibrosis suggesting post-necrotic scarring. Haematoxylin-eosin. × 20.

FIG. 3.—Section from a hepatocellular carcinoma showing merging of neoplastic and non-neoplastic trabeculae. Haematoxylin-eosin. × 75.

FIG. 4.—Section of an adenoid hepatocellular carcinoma. Although superficially resembling a cholangiocellular carcinoma these acini contain bile-stained material and the tumour cells can accordingly be regarded as bile producing, which places the neoplasm in the hepatocellular group. Haematoxylin-eosin. × 70.

FIG. 5.—Unusual case of hepatocellular carcinoma arising in a liver in which the parenchyma is almost completely destroyed and replaced by collapsed stroma with numerous newly-formed bile ducts. Despite the marked hyperplasia of the latter the tumour is hepatocellular. Haematoxylin-eosin. × 25.

FIG. 6.—Mitochondria in normal liver. Many appear grey in the photograph since they are slightly out of focus. P.A.H. × 450.

FIG. 7.—Mitochondria showing no significant change in cells of hyperplastic parenchymal nodule. Biopsy of this liver two months later demonstrated a typical hepatocellular carcinoma. P.A.H. × 450.

FIG. 8.—Hepatocellular carcinoma showing almost complete absence of mitochondria. The poorly-stained bodies in the cytoplasm suggest possible degenerated forms. P.A.H. × 450.

FIG. 9.—Numerous polyploid cells in liver nodule in region of hepatocellular tumour. Haematoxylin-eosin. × 90.

FIG. 10.—Large " hour-glass " nucleus in cell of hyperplastic liver nodule suggesting amitotic division. No spindle could be observed in this and other similar cells. Haematoxylin-eosin. × 750.

FIG. 11.—Liver biopsy from Bantu male, aged 21 years, showing the histological features of an infective hepatitis of some weeks duration. At post-mortem one month later a large hepatocellular carcinoma was present. Haematoxylin-eosin. × 70.

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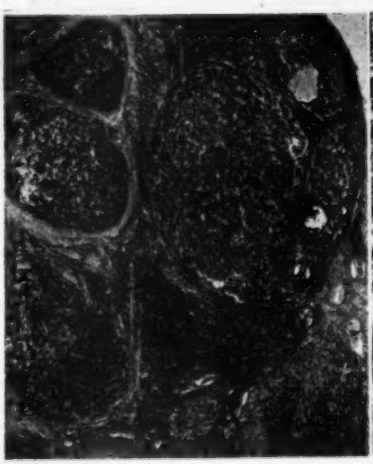

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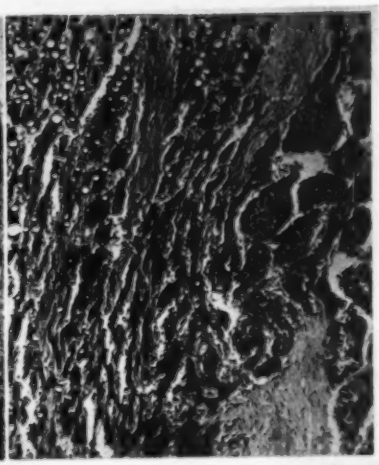

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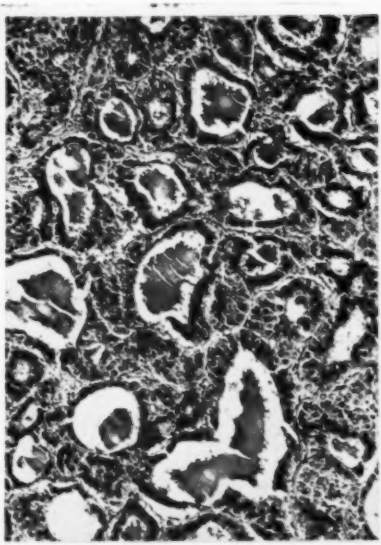

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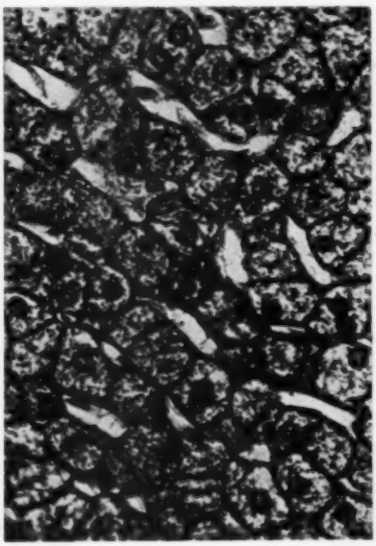

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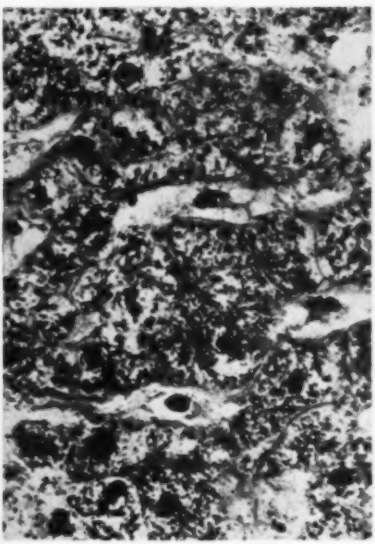

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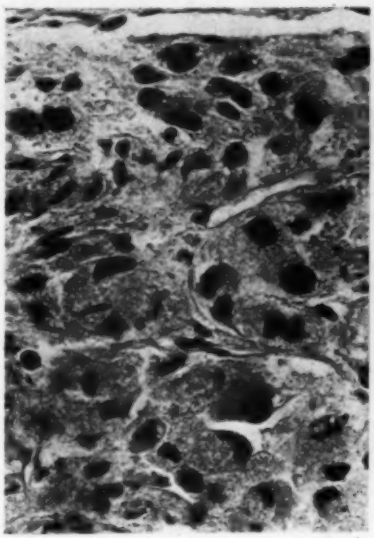

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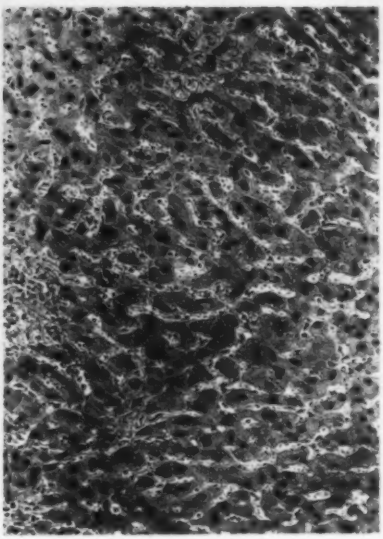

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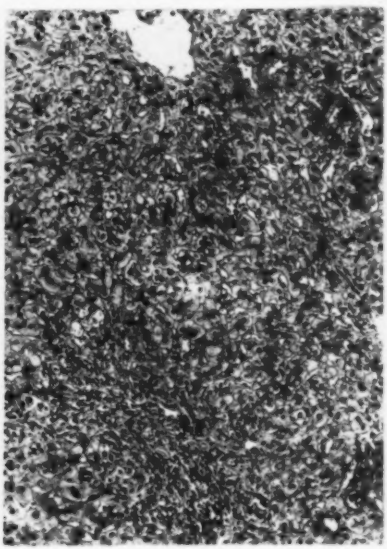

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TABLE I.—*Distribution of 88 Primary Liver Carcinomas According to Cell Type*

Cell type.		Male.		Female.	Total.
Hepatocellular	(*a*) 44	73		3 7	80
	(*b*) 29			4	
Cholangiocellular	(*a*) 1	2		2 2	4
	(*b*) 1			0	
Unclassified	(*a*) 2	4		0 0	4
	(*b*) 2			0	
Total		79		9	88

(*a*) Tumours examined at post-mortem.
(*b*) Tumours examined by liver biopsy.

Utilising the preliminary cancer rates available for the peri-urban native townships of Johannesburg, the frequency of each cell type was calculated by applying the ratios found in this study to the total number of liver cancers observed at the cancer registry of this Institute in 1953 from the Johannesburgh urban area. Similarly, the ratios obtained by Edmondson and Steiner (1954) were applied to the number of liver cancers which would be expected from an American population of similar age and sex composition calculated according to rates put at our disposal by Dr. H. Dorn of Bethesda. The results are presented in Table II and it would appear that the increased incidence of primary liver cancer in this area is due mainly to an increase in the hepatocellular type and not in the cholangiocellular type. Hong Kong is the only region where an increased frequency of primary liver carcinoma appears due to an increase in the cholangiocellular type of tumour (Hou, 1955). These tumours are, however, unusual in that they are frequently associated with a marked bile duct proliferation caused by infestation with *Clonorchis sinensis*.

TABLE II.—*Frequency of Cell Types as Calculated from the Observed Number of Cases of Liver Cancer in the Peri-urban Townships, Johannesburg* (1953), *and the expected Number of Cases Based on Incidence Rates for the Metropolitan Areas, United States,* 1947–48

	Males.		Females.	
Cell type.	Number observed in S.A. Bantu.	Number expected U.S. White.	Number observed in S.A. Bantu.	Number expected U.S. White.
Total	30	2·9	10	2·0
Hepatocellular	27·7	2·6	7·7	1·1
Cholangiocellular	0·8	0·3	2·2	0·9
Unclassified	1·6	—	—	—

True mixed tumours of both cell types would appear very rare in the Bantu Negro although the presence of adenoid areas in hepatocellular tumours may simulate such neoplasms.

In all countries, the hepatocellular form is more frequently associated with cirrhosis than the cholangiocellular type (Higginson, 1955). Further, the latter type is proportionately more frequent in females than in males, a fact which would appear analogous to the higher incidence of cancers of the extra-hepatic biliary system reported in this sex. It is accordingly of interest that no increased frequency of other tumours of the biliary system i.e. gall bladder, common bile duct and head

of the pancreas, has been observed in the South African Bantu (Higginson, 1951). These observations would suggest the possibility that the aetiology of the cholangiocellular form is more closely allied to tumours of the extra-hepatic system than to those of the liver parenchyma.

In view of these facts, it would seem advisable to regard this histological distinction as of probable fundamental significance which should be made in all papers reporting the incidence of liver carcinoma. It is probable that the aetiological factors for each cell type are different and that the appearance of a specific cell type is dependent on the environmental context in which the cancer develops whether internal or external.

In contrast to the high incidence of carcinoma in adults, the absence of any increase in the African region of hepatoblastomas (embryonic carcinomas) in children is striking, and in over 3000 post-mortems and 3000 cancers in Bantu patients, I have seen no tumour of this type. This would suggest that there is no connection between this type of growth and the factors causing malignant hepatoma in adults.

A. HEPATOCELLULAR CARCINOMA

1. *Cytological Changes*

(a) *Cytoplasmic changes*

Well differentiated hepatocellular tumours retain certain of the cellular characteristics of normal liver, as is seen in the ability to store glycogen, and form bile (Berman, 1951 ; Edmondson and Steiner, 1954). The various forms of degeneration which are found in non-neoplastic liver cells can also be observed in tumour cells. Similar cytological features have been noted in experimental liver-cell carcinomas, although bile formation is very rare (Firminger, 1955).

The advent of electron microscopy and improved methods of isolation has led to a renewed interest of recent years in the alterations of the mitochondria of malignant cells (Cowdry, 1955). Howatson and Ham (1954) demonstrated a decrease in the number of mitochondria in two experimental rat hepatomas studied by electron microscopy, and Price *et al.* (1952) have described alterations in the mitochondria of the precancerous liver.

The majority of tumours examined in the present series showed a reduction in the number of mitochondria in many cells although not in all (Fig. 6–8). The remaining mitochondria often appeared coarser and more spherical than normal, and filamentous forms were rare. In addition in many cells, although only scanty mitochondria could be found, poorly staining spherical or oval bodies were seen, the appearance of which suggested possible degenerated forms. Well-stained mitochondria in other portions of the same section suggested that this picture was not an artefact. Morphological changes in the mitochondria of non-neoplastic cells from cancerous livers were equivocal (Fig. 7). It is possible, however, that better methods may demonstrate morphological and chemical changes in the mitochondria of the premalignant human liver similar to those demonstrated in rats.

(b) *Nuclear changes*

The various nuclear forms observed in cancerous and non-cancerous livers in the West African native have been reviewed by Roulet (1951), and my observations have been similar.

(i) *Binucleate cells.*—Gillman (1940) reported a high frequency of binucleate and multinucleate cells in Bantu livers, but was unable to obtain material from European subjects for comparison. His figure of 20 per cent was derived from a total count of 4000 cells, but he does not state how many individuals were involved. Using the same method we found that in general, the number of binucleate and multinucleate cells in the non-cirrhotic Bantu liver was of the same order as in non-cirrhotic livers from South African white subjects (Table III). One liver from a Bantu patient was exceptional in that 40 per cent of the cells were binucleates, but no cause for this increase could be suggested. Contrary to expectation no increase in binucleate cells could be demonstrated in cirrhotic livers with cancer.

TABLE III.—*Percentage of Binucleate Cells and Mitoses in Bantu and European Livers*

Type of liver.	Number of livers.	Number of cells.	Binucleate or multinucleate cells %.	Mitosis %.
Non-cirrhotic European livers	8	6525	9·3 (5·8–13·3)	0·02
Non-cirrhotic Bantu livers—				
From post-mortem*	8	8048	9·4 (5·5–13·5)	0·01
From biopsy	10	2690	7·8 (4–12)	0·07
Cirrhotic Bantu liver with cancer	8	7566	8·9 (6·4–12·5)	0·00

* One liver in which the percentage of binucleates was 40 per cent was excluded as being definitely abnormal. No cause for the high percentage of binucleate cells could be postulated in this case.

The method used, however, is limited by variations introduced by section thickness, the size of cell, and nucleo-cytoplasmic ratio, so the results have not an absolute value.

Further, since there may be considerable variation in the number of binucleate cells in different parts of the liver lobule, it is possible that differences may be demonstrable by counting a much larger number of cells. I feel, however, that with the above qualifications there is little evidence of a *marked* general increase in binucleate cells in South African Bantu subjects.

(ii) *Polyploid nuclei.*—In well-differentiated tumours the nuclei may be small and regular. In others, the nuclei are large with prominent nucleoli and nuclear membranes, and in many cases giant multinucleate forms are present. Furthermore the cells of hyperplastic non-malignant liver nodules surrounding the tumour may also show marked nuclear variation in size, many being greatly enlarged with prominent nucleoli (Fig. 9). While an increase in nuclear size as expressed by measurement in one diameter does not necessarily imply polyploidy, the increase in size and density of basophilic staining associated with apparent amitotic division into two nuclei of normal size (*vide infra*) does suggest that polyploidy is a common feature of these livers. Such nuclear changes are not, however, confined to livers with cancer but may be observed in many other conditions.

(iii) *Occurrence of mitosis and amitosis.*—From Table III it would appear that mitosis in the normal cell or even in the hyperplastic nodule of the cirrhotic liver is very uncommon.

Muir (1908) commenting on the infrequency of mitosis in the nodular hyperplastic liver stated that he himself had never observed this type of cell division. In over 70 cirrhotic livers seen by me from consecutive post-mortems mitotic

figures appeared almost completely absent and in only one liver were they numerous. In this case many amoebic abscesses were present. On the other hand mitoses are easily demonstrated in malignant liver tissue.

The failure to demonstrate mitosis readily in normal or cirrhotic livers may be the result of several variables including prolonged interphase, short mitotic time and changes in mitotic periodicity. There is experimental evidence, moreover, to show that mitotic time in tumour tissue is increased and that the interphase period is shortened (Cowdry, 1955).

Another possibility should, however, be considered. Milne (1909) after a review of the earlier literature in conjunction with his own material concluded that amitosis was a usual procedure in liver cell multiplication. Although this hypothesis is now held in disrepute and it is generally stated that amitosis is rare in man (Cameron, 1952 ; Cowdry, 1955), the morphological evidence in this and other studies on liver cancer suggests it merits further attention. The pathological features of many hyperplastic nodules in cirrhotic livers with or without primary carcinoma in our material suggested rapid growth, but mitosis in these nodules could not readily be demonstrated, On the other hand, all gradations between large polyploid nuclei, oval, constricted or " hour-glass " nuclei, and cells with two closely-approximated nuclei could be found as described by Milne (1909) giving the impression of direct division (Fig. 9, 10). If these morphological changes which were seen in both biopsy and post-mortem material are not artefacts, it is difficult to accept any other explanation except that they represent stages in amitotic cell division. In certain conditions, however, such as in infective hepatitis, occasional cirrhotic livers, and hepatic carcinoma, cell division would appear to be predominantly mitotic. It would appear accordingly worth while to re-investigate the relative significance of direct and indirect division in liver cell multiplication and its relationship to hepatic carcinogenesis. In the rat, however, contrary to experience in man mitotic figures are observed without difficulty in the regenerative or hyperplastic parenchymal nodules, which precede experimental hepatoma development (Firminger, 1955).

Significance of cytological changes

The significance of these cytoplasmic and nuclear changes is unknown, but they are of interest in view of the hypothesis that malignant tumour formation " involves an essentially regressive change with loss of genetic protein, enzyme or antigenic properties " (Haddow, 1955). Evidence for such regression in liver tumours in relation to the binding of " butter yellow ", is given by Miller and Miller (1953). Weiler (1952) has also shown differences in the antigenic properties of the liver associated with the mitochondria. Further, Hauschka and Levan (1953) have shown that cell specificity is inversely related to the degree of polyploidy. While it may not be possible to state with certainty that the above changes are significant morphological evidence for similar alterations in man, they are not contradictory.

2. *Cirrhosis*

(a) *Frequency of malignant neoplastic development in cirrhosis*

The association of cirrhosis and primary liver carcinoma is now well established, but the extent of this association varies. In Europe and North America approxi-

mately 3 to 12 per cent of cirrhotic livers at post-mortem show malignant neoplasia. On the other hand, in Africa and Indonesia, where the incidence of liver cancer is high, up to 50 per cent of cirrhotic livers at post-mortem may show malignant change, especially of the hepatocellular type (Higginson, 1955). The proportion of *non-cirrhotic* livers developing carcinomatous change is approximately of the same order for all countries.

(b) *Type of cirrhosis*

In America and Europe liver cancer is usually described as arising in livers with a diffuse septal cirrhosis (" portal or Laennec's cirrhosis "), often believed to follow severe fatty change. Isolated reports do, however, refer to cancer arising in post-necrotic cirrhosis. In the South African Bantu, on the other hand, hepatocellular carcinoma usually arises in a severe cirrhosis with hyperplastic parenchymal nodules the histological features of which in many cases correspond to those described as characteristic of post-necrotic scarring (Higginson, Grobbelaar and Walker, 1956).

(c) *Significance of parenchymal cell hyperplasia in the development of hepatocellular carcinoma*

Muir (1908) ascribed to the view first proposed by Orth that hepatocellular liver cancer arose as a result of excessive parenchymal hyperplasia. He described the merging of neoplastic and non-neoplastic liver nodules, an appearance which could be duplicated in many of the present livers (Fig. 1–3).

In addition to the severe cirrhosis with hyperplasia in which carcinoma arises (*vide supra*) many livers from adult Bantu subjects show a diffuse portal fibrosis often associated with marked haemosiderin deposition and which may develop into a septal cirrhosis. The aetiology of this lesion is as yet unestablished (Higginson, Grobbelaar and Walker, 1956). Parenchymal cell hyperplasia is not, however, a major feature of this type of lesion and the histological picture is that of a chronic progressive interstitial hepatitis.

In Table IV the frequency with which carcinoma develops in each type of liver lesion is shown in a series of 876 necropsies. In contrast to the severe cirrhosis with hyperplasia neoplasia is rare in livers with only fibrosis or cirrhosis in which hyperplasia is minimal or absent. The associated liver lesions observed in 50 carcinomas are also shown in Table V.

TABLE IV.—*Association of Liver Cancer and Type of Liver Lesion*

Liver lesion.	Males.		Females.	
	Livers.*	With cancer.	Livers.*	With cancer.
Non-fibrotic	271	0 (0·0%)	260	0 (0·0%)
Slight fibrosis	142	1 (0·7%)	55	2 (3·6%)
Moderate fibrosis	50	0 (0·0%)	17	0 (0·0%)
Severe fibrosis (fine cirrhosis)	10	1 (10%)	5	0 (0·0%)
Severe cirrhosis with hyperplasia.	39	21 (54%)	9	0 (0·0%)
Total	512	23	346	2

* These livers were part of a series of 876 consecutive post-mortems. Livers with necrosis, cardiac cirrhosis and other miscellaneous lesions have been excluded; also, one liver in which tumour infiltration was so great as to permit no conclusion on the type of associated liver lesion.

TABLE V.—*Liver Lesions Observed in 50 Post-mortem Cancers*

	*Males.		Females.	
	Liver cell type.	Bile duct cell type.	Liver cell type.	Bile duct cell type.
Nodular hyperplastic cirrhosis suggesting post-necrotic or post-hepatitic origin	34	1	3	0
Fine cirrhosis. Aetiology uncertain.	1	0	0	0
Portal or focal fibrosis (suggesting post-hepatitic origin)	4	0	0	2
Normal liver	1	0	0	0
Insufficient non-cancerous liver	4	0	0	0
	44	1	3	2

* Two livers of uncertain cell type were excluded.

These observations support the view held by many workers, that in man neoplasia is related to parenchymal cell hyperplasia and may represent an exaggerated response. Since in man liver cell hyperplasia is most frequently observed in cirrhosis, a logical explanation is available for the frequent association of cirrhosis and cancer. It is theoretically possible that all cirrhotic livers possess the potentiality of malignant neoplasia if survival is sufficiently long and such a hypothesis has been favoured by Edmondson and Steiner (1954) to explain the high frequency of liver carcinoma in idiopathic haemochromatosis. If this is so, it would appear that in the European and American liver the progress from hyperplasia to neoplasia is slow so that death usually results from other causes such as oesophageal varices or liver failure. In contrast in the South African Bantu neoplasia is of rapid development so that death is usually due to liver carcinoma.

(d) *Is cirrhosis part of the neoplastic process in the African liver?*

In white races a clinical history of cirrhosis of several years duration is not uncommon before carcinoma is diagnosed, but among Bantu patients, a history of over a few weeks is exceptional. All Berman's (1951) cases were in Negro mine workers who had passed several medical examinations prior to acceptance for work, and it is unlikely that obvious clinical signs of cirrhosis should have been missed, yet at post-mortem, cirrhosis was present in all. It would appear logical accordingly to regard the development of cirrhosis with severe damage in the liver of the African Negro as an immediate precursor of malignancy.

(e) *Cancer in non-cirrhotic livers*

If parenchymal cell hyperplasia in cirrhosis is regarded as an essential precancerous lesion in man, it is necessary to explain those tumours which develop in "non-cirrhotic livers", of which there were 7 in the present series (Table V). In only one of these cases did the surrounding liver appear undiseased in the sections examined. In the remainder, the liver showed irregular fibrosis and inflammation of the portal triads with foci of new bile duct formation. In some parts, the parenchyma appeared normal but elsewhere foci of regenerating liver cords were present. The histological features in these cases were those of a low-grade sub-acute or chronic hepatitis. These findings would suggest that these

cancers also were arising in abnormal livers which were the seat of inflammation and regenerative change. Of these 6 livers, 2 were in females and associated with cholangiocellular carcinoma.

(f) *Unicentric as opposed to multicentric origin*

The histological picture and the frequency with which nodular hyperplastic cirrhosis in the Bantu merges into primary hepatocellular carcinoma would suggest that the basic neoplastic change is multicentric. It is probable, however, that malignant neoplasia in one focus develops sooner than in others, giving the appearance of unicentric origin or so-called " massive " cancer. In some livers, the pathological picture suggested that several nodules were undergoing simultaneous malignant change. If so, the prospects of surgical intervention in hepatocellular carcinoma are poor, since other potentially precancerous foci will still remain in the organ.

(g) *Diagnostic significance of cirrhosis*

Many African patients with hepatomegally are admitted to hospital, in whom liver biopsy shows no neoplastic tissue. Since few cases of carcinoma develop in non-cirrhotic livers, the absence of cirrhosis should always stimulate search for other causes of liver enlargement before accepting the diagnosis of liver carcinoma. The finding of a nodular cirrhosis with marked polyploidy and hyperplasia in a liver biopsy from a Bantu patient, is grounds, however, for suggesting to the clinician the probability that a carcinoma either exists or will develop in such a liver.

Conclusions on cirrhosis and hepatocellular liver cancer in Africa

To summarise, it would appear that in man hepatocellular carcinoma arises in the parenchymal hyperplastic nodules of a cirrhotic liver, and liver-cell hyperplasia in cirrhosis can thus be regarded as a potentially precancerous lesion. To explain the different incidence in various races it is necessary to postulate that these changes are more intense and rapid in the Bantu cirrhotic liver than in the cirrhotic liver of the white race.

Under certain circumstances, zonal or focal hepatic necrosis is followed by complete restitution of parenchyma. If, however, necrosis is massive, or considerable collapse and scarring have occurred, controlled regeneration merges into hyperplasia with loss of the normal architecture as seen in cirrhosis. It is in livers with hyperplasia that carcinoma of the hepatocellular type develops, and it would appear advisable to distinguish between regeneration or restitution of the normal, and hyperplasia, which in the liver should be regarded as a precancerous lesion, at least in the African Negro.

3. *Relationship of Human and Animal Hepatocellular Tumours*

Tumours of the liver have been produced by a wide variety of agents, both chemical and dietary in experimental animals. Although cholangiocellular tumours are more frequent in animals than in man, hepatocellular tumours predominate (Firminger, 1955). Not only is it impossible to distinguish between tumours produced by different chemical agents in different species but in addition there would appear to be fairly general agreement that hepatocellular tumours are

preceded by parenchymal cell hyperplasia (Firminger, 1955) whether the result of necrosis, fatty change, or cirrhosis. Although the significance of animal hepatic carcinogens has yet to be demonstrated in man it is our opinion contrary to that of Davies (1952, 1955) that it is reasonable to consider the basic pathological process in man and animals as similar, and to regard excessive parenchymal liver-cell hyperplasia, however caused, as of primary importance in hepatoma formation. For this reason the study of experimental hepatomas may prove of value in elucidating the pathogenesis of human liver carcinoma.

B. CHOLANGIOCELLULAR CARCINOMA

The cholangiocellular form of liver carcinoma is comparatively rare in the South African Negro, in whom, as in other races it is relatively more frequent in females (Table I). In animals, foci of bile duct hyperplasia and dilatation (cholangiofibrosis) would appear to be the usual precursory lesions to cholangioma (Firminger, 1955). Apart from the adenocarcinomas arising in foci of bile duct hyperplasia associated with *Clonorchis sinensis*, such a sequence is rare in man. Nevertheless, the rarity of this tumour is of interest and merits investigation, in view of the degree of new bile duct formation seen in many cirrhotic livers in the Bantu, and which may be more conspicuous than liver cell hyperplasia (Fig. 5). Although these new bile ducts may represent hyperplasia of the cholangioles, this observation lends further support to the view that cholangiocellular and hepatocellular tumours should be regarded as separate entities.

C. AETIOLOGICAL BACKGROUND OF LIVER CARCINOMA IN AFRICA

Differences in racial frequency of carcinoma imply either different carcinogenic stimuli or different sensitivity of the liver to such stimuli. Even although unknown, the possible nature of such carcinogenic stimuli may be considered. Such stimuli must be either intrinsic, extrinsic or a combination of both.

Intrinsic stimulus.—In view of the relative freedom from liver carcinoma of the American Negro, such a stimulus must be the result of hostile environmental circumstances and thus ultimately dependent on external environment.

Extrinsic stimulus.—Such stimuli are either (*a*) equally effective in livers of all races (and hence the level varies in different communities) or (*b*) their effects are modified by the state of the liver.

Nature of Possible Extrinsic Carcinogenic Stimuli

(a) *Parasites.*—The two common parasites which have been implicated as aetiological factors in liver cancer in Africa are malaria and bilharziasis. In the Johannesburg area, malaria is not endemic and yet all reports show that primary liver cancer is frequent. While among males, 25 per cent show evidence of bilharzial infestation especially *Sch. haemotobium*, no correlation between this parasite and primary liver cancer has been demonstrated (Higginson and de Meillon, 1955). Hou (1955) has discussed the relationship of *Clonorchus sinensis* to adenocarcinoma of the liver in the Chinese. It is true that this parasite may be a factor in certain regions, but Chinese who live in regions where this parasite is absent also show a high frequency of liver carcinoma (Marsden, 1955). This would suggest that the liver in the Chinese is basically susceptible to different external carcinogenic stimuli, one of which may be *Clonorchus sinensis*.

(b) *Siderosis.*—Haemosiderin deposits are frequently found in the livers of Negro patients in many parts of Africa, but no increased association of siderosis and liver cancer has been demonstrated (Higginson, 1955). There would, however, appear to be an increased frequency of liver cancer in cases of haemochromatosis among white races, although this is unlikely to be the direct result of iron deposition (Edmondson and Steiner, 1954).

(c) *Native medicines.*—The significance of native medicines among the Bantu is unknown, although widely ingested. Grusin, (1955) has demonstrated that in Johannesburg, potassium bichromate, a possible carcinogen, is widely used as an emetic by healthy individuals. Experimentally the *senecio alkaloids* have been implicated as producing hepatomas in mice (Hunt and Schoental, 1952), and cases of acute senecio poisoning have been reported from South Africa. In Jamaica such alkaloids have been implicated in liver disease, but no increase in liver cancer has been reported (Bras, Brooks and Depass, 1954). It is clear, however, that a careful systematic analysis of native medicines must be made for carcinogenic agents, although such drugs are unlikely to be sufficiently widespread to be of equal importance in all localities.

(d) *Hepatotoxic viruses.*—The only disease in man which has been directly implicated as a cause of liver cancer is infective hepatitis. Cases have been reported from West Africa describing the transition of infective hepatitis to carcinoma (Bergeret and Roulet, 1947) and a similar sequence has been reported in England (Walsh and Wolff, 1952). In Johannesburg infective hepatitis is not uncommon and from 1951 to 1954, 134 clinically diagnosed cases were seen at Baragwanath Hospital. Cases of acute massive necrosis were also seen in which the histology was consistent with fulminating viral hepatitis. In many livers in this region with primary cancer, the pathological picture of the associated cirrhosis was consistent with being the sequel to infective hepatitis. Further we have observed cases of infective hepatitis as demonstrated at biopsy in which liver cancer has later been found at post-mortem (Fig. 11). While this disease has not yet been proved to be a carcinogenic stimulus the hypothesis has considerable support.

Although the levels of these stimuli may vary in the environment, many are widespread among communities in which liver cancer appears to be uncommon. It is reasonable, therefore, to suggest that if such stimuli are in fact implicated their effect depends on alterations in liver metabolism which render the organ susceptible to the action of external factors, a possibility which has been discussed by Gillman and Gillman (1951). Evidence for abnormalities in hepatic metabolism are discussed in the following section.

Hepatic Dysfunction in the Indigenous African Negro

Evidence in man and animals suggests that an already damaged liver is more susceptible to hepatotoxic agents than a healthy liver. The pathological and physiological abnormalities observed in the livers of the local Bantu and their significance have been discussed in detail elsewhere (Higginson, Grobbelaar and Walker, 1956).

It would appear that while the liver histology, "liver function tests" and serum proteins are normal in African babies, irreversible abnormalities arise during the first years of life as compared to European infants. Further, all necropsy and "liver function" series show a high incidence of hepatic disorder in apparently healthy

adult Africans which appears irreversible (Wayburne, Bersohn and Sussman, 1953 ; Walker and Arvidsson, 1954). Although these changes may only reflect relatively slight liver damage, and accordingly cannot *per se* be regarded as premalignant, we feel there is evidence to suggest that such livers may be unduly susceptible to hepatotoxic agents causing cirrhosis of the post-necrotic or premalignant type (Higginson, Grobbelaar and Walker, 1956). It is also possible as has been suggested by Davies (1949) that the hormonal background of the African Negro may be abnormal due to either relative or absolute hyperoestrogenation. This fact may be of significance in view of the effects of hormones in experimental hepatomas. While it is impossible to prove that the above abnormalities do actually sensitise the liver to carcinogenic agents, they provide evidence that the metabolism of the liver in the African Negro by Western standards is abnormal.

ROLE OF MALNUTRITION

The number, cell type, and induction time of many experimental animal tumours can be considerably modified by dietary manipulation (Opie, 1944). Liver tumours have also been produced in rats by a choline deficient diet alone (Salmon and Copeland, 1954). Many communications accordingly on liver disease in Africa have implicated malnutrition, especially deficiencies of protein and B complex, as a direct cause of cancer (Berman, 1951). On the other hand, there is little direct evidence to show that deficiencies of those food factors, the absence of which causes liver disease in animals, are involved in human liver disease (Higginson, Grobbelaar and Walker, 1956). In man, only in kwashiorkor, a nutritional disease of infancy, have the liver lesions been definitely proved to be related to malnutrition. There is no satisfactory support for the view that the post-necrotic cirrhosis of the adult South African Bantu is a result of this disease or any other specific dietary deficiency. Furthermore, in the adult Bantu Negro, cirrhosis following severe fatty change is very rare, a fact which would suggest that a deficiency of lipotropic agents is not important.

While the metabolic abnormalities in infancy, described above, may be the result of sub-clinical malnutrition, those seen in adult life appear irreversible and do not disappear in an improved dietary context (Wayburne, Bersohn and Sussman, 1952, 1953). The geographical distribution of malnutrition even in infancy, is not identical with that of liver carcinoma. Hepatic carcinoma appears rare in India (Khanolkar, 1951) although malnutrition is widespread (Thomson, 1946). In Jamaica, where nutritional deficiencies in both children and adults may be marked, liver cancer is uncommon (Bras, Brooks and Depass, 1955). Further, I have been unable to find reports of an increase of primary liver carcinoma from those parts of Europe where undernutrition was widespread in World War II. It is unlikely, therefore, that malnutrition whether of infancy or adult life, alone is capable of inducing liver tumour formation. If, as has been suggested, the metabolic disorders of infancy predispose the liver to carcinogens in later life the role of malnutrition would, at the most, be indirect.

CONCLUSION

While the pathogenesis of primary liver cancer in South Africa would appear to have many similarities to hepatocellular tumours produced by " butter yellow " in rats, the possibility and significance of irreversible changes in liver metabolism

arising in infancy warrants further study. If, as is possible, these abnormalities sensitise the liver to carcinogens in later life, then prevention of this cancer is possible. The significance of child welfare in such regions accordingly assumes basic importance, since the identification and elimination of the extrinsic carcinogenic stimuli may prove difficult or impossible, especially if viral in origin.

SUMMARY

Certain pathological and cytological features of liver carcinoma on the African continent are reviewed.

It is suggested that the distinction between hepatocellular and cholangiocellular forms may be of fundamental significance. The high incidence of liver cancer in the Bantu is mainly due to an increase in the former.

The relationship of cirrhosis to primary liver cancer is briefly discussed and it is emphasised that in Africa it is a nodular hyperplastic cirrhosis probably of post-necrotic origin which most frequently becomes malignant, in contrast to the type of cirrhosis described in Europe and North America. It is considered that parenchymal cell hyperplasia associated with cirrhosis is essentially precancerous.

It is suggested that the liver in the African native is damaged in childhood, thus rendering it more susceptible to a carcinogenic stimulus. The significance of malnutrition would accordingly be only indirect by sensitising the adult liver. The only carcinogenic stimulus as yet implicated in human liver cancer is infective hepatitis, although many other possibilities should be considered, especially native medicines.

Prevention would appear to be possible either by eliminating abnormalities in hepatic metabolism arising in infancy or by avoidance of the carcinogenic stimuli causing post-necrotic cirrhosis in later life.

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A STUDY OF TUBERCULOSIS AND CANCER MORTALITY RATES WITH SPECIAL REFERENCE TO LUNG CANCER RATES

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Of all the factors which may have aetiological significance in connection with lung cancer, only two receive popular attention. These are smoking and residence in cities. The reason why the latter is associated with a greater increase of lung cancer than living in the country, is most probably concerned with the inhalation of carcinogens, released by the consumption of fuels of all types. The concentration of atmospheric carcinogens, as revealed by air filtration studies, has been shown to be higher in the larger cities than in rural areas. (Stocks and Campbell, 1955). Much suspicion attaches to petrol and diesel engines, tarred roads and rubber tyres, but little evidence directly incriminating them has been discovered.

Today, so great is the interest in smoking and residence in cities that there is danger of attention being deviated from other important aetiological factors.

Two examples may clarify this. Dublin, Bristol, Leicester, Nottingham and Sheffield have populations of comparable size and density (Table I). Up to the age of 64 the lung cancer rate for males is similar in these cities (Fig. 1),

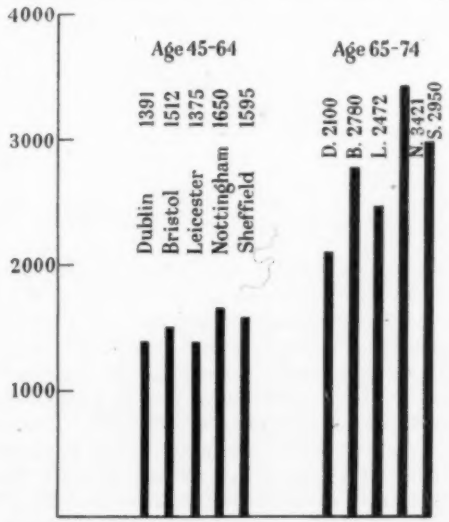

Fig. 1.—Mortality rates per million living, for males, for respiratory cancer at age group 45/64 and 65/74 in Dublin, Bristol, Leicester, Nottingham and Sheffield (average 1950–1953).

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but there are marked differences in the fuel consumptions. For instance, the annual consumption of coal for Sheffield is more than ten times that of Dublin (1954, Sheffield 4,385,000 tons, Dublin 437,634 tons) and in 1938 the Sheffield Corporation Transport Commission alone burnt as much diesel oil as the whole of the Irish republic. (Ministry of Fuel and Power, 1952).

TABLE I.—*Acreages and Density of Population of County Boroughs*

County Borough.	Population.	Acreage.	Persons per acre.
Dublin	522,183	21,907	24
Bristol	442,944	26,550	17
Leicester	285,181	16,987	17
Nottingham	306,055	16,117	19
Sheffield	512,850	39,586	13

As the disease rates of Dublin and Sheffield are comparable it suggests that either the method of burning is of great importance or that other factors influence the cancer producing properties of fuels.

The cigarette consumption of the two populations may be about the same (Table II), and this might be regarded as evidence in favour of the importance of smoking as a cancer factor, but comparison of the American and English figures of tobacco consumption and lung cancer rates raise some problems difficult of solution if tobacco be regarded as a major causal factor, without reference to other possible factors.

TABLE II.—*Tobacco Statistics* (from "*Tobacco Statistics*", Journal of the Royal Statistical Society, 1952).

	1939.		1949.	
Country.	Number of cigarettes per adult.	Number of lb. tobacco per adult.	Number of cigarettes per adult.	Number of lb. tobacco per adult.
America	1750	8·5	3188	10·1
Denmark	612	7·6	894	6·0
Eire	1413	4·8	2308	6·5
United Kingdom	1955	5·3	2029	5·5

"Number of lb. tobacco per adult" here *includes* weight of cigarettes.

For upwards of 15 years (Table II), the American consumption of all tobacco products per head of the adult population has been almost twice that of adults in Britain. It is reasonable to suppose, therefore, that for 20 years and probably for 30 years or even longer, Americans have smoked at least as much as Britons. It is true that in 1940 the cigarette consumption in Britain was slightly greater than in America—2000 to 1750 respectively—but in view of the early mechanisation and industrialisation of the larger American cities, it would again be reasonable to expect the lung cancer rates of America to be equal or even greater than those of Britain. Gilliam (1955) stated that in 1940 in American cities of 1,000,000 and over the lung cancer rate amongst white males was 288. This was less than the Rural England rate—300—and less than half the rate in Greater London—650. This marked difference in the incidence of the disease in the two countries is very interesting, especially when it is considered that the cigarette factor probably operates with equal intensity in both.

It seems possible, therefore, that unless other important and unknown factors are operating, the effect of smoking is minimised.

The object of this paper is to advance some points in support of the theory that tuberculosis and cancer are related, and that there may be an association between extra-pulmonary forms of tuberculosis and lung cancer.

In most Western civilised countries the mortality rate for all forms of tuberculosis has for many decades now been steadily falling. In England, Denmark and America, for instance, these are critical days for tuberculosis, for if the graph of the mortality rate for males, per million living, continues falling at its present rate, the zero line should be reached about 1960. Whether this represents the lowest point of the trough of one of those great and slowly moving waves of tuberculosis which have spread over Europe during past centuries, and whether the mortality rate will in future decades commence rising again remains to be seen.

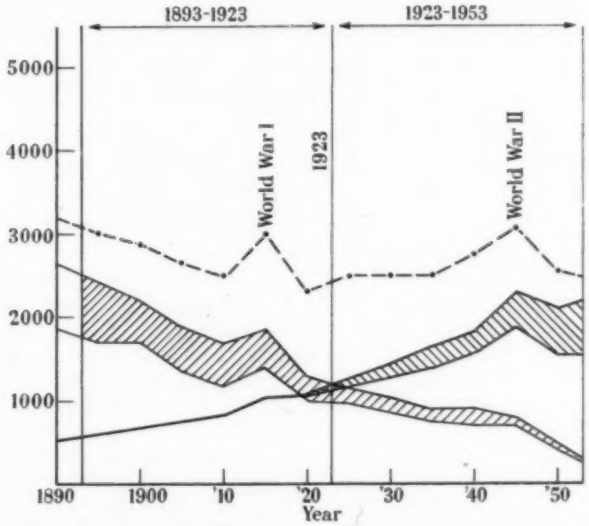

FIG. 2.—Mortality rates per million living, for males, showing combined rate (1), all forms of tuberculosis (2), pulmonary tuberculosis (3), all forms of cancer (4) and non-respiratory cancer (5) from 1893 to 1953.

Extra pulmonary tuberculosis. Respiratory cancer.

Fig. 2 shows this graph for males in England and Wales. It will be observed that there is a sharp peak produced by the 1914–1918 war and another much less pronounced during the 1939–1945 war.

1923 was in Great Britain an important year for males, for then the mortality rate for tuberculosis and cancer equalled each other : about 1200 each. Now if the mortality rate for all forms of cancer be plotted (Fig. 2), it will be observed that in 1953, i.e. 30 years after 1923, the rate was about 2250 and this approximates to the tuberculosis mortality rate of 2400, 30 years before 1923,

i.e. in 1893. In other words during the past 60 years there appears to have been an almost exact replacement of the mortality rate of tuberculosis by cancer.

There are several explanations of this phenomenon.

(1) It may be the result of ageing of the population which has gone on steadily over the past half century, and this would account for more people reaching cancer age.

(2) Possibly a similar relationship could be shown between tuberculosis and other diseases such as coronary heart disease or between typhoid and cancer.

(3) The relationship may be entirely fortuitous and the fact that similar graphs for females do not show the close approximations lends support to this view.

(4) On the other hand this simultaneous waxing of the one disease and waning of the other may be evidence of a relationship between them, the nature of which is unknown. This was the view of Cherry (1924, 1925, 1933) and it was summarised by Cruikshank (1939) in his very detailed and important paper, " The Association of Tuberculosis and Cancer, or the Theory of Cancer Phage ". In it he says :

> "In a series of papers on the statistical relationship of phthisis and cancer, Cherry has demonstrated the following (England and Wales) :
>
> A. (1) For a period of four census units the crude ratio
>
> $$\frac{\text{deaths from phthisis and cancer}}{\text{deaths from all causes}}$$
>
> from age 15 to 75+ has remained practically constant both for males and females (20% ratio).
>
> (2) Correction of this crude ratio for (*a*) accidental deaths, and (*b*) improved methods of diagnosis in recent years, has the effect of sharpening the relation.
>
> (3) From the way in which the data are extracted in Census Units the constancy of this ratio means that over the period covered decrease of deaths from phthisis has been *exactly* compensated by increase of deaths from cancer.
>
> (4) This relationship of the two diseases is unique. " There is no other pair of important diseases in which a similar movement can be detected in the rates."
>
> B. (1) The total aggregate percentage rates of the two diseases in the census units has remained fairly constant, the decreasing rate for phthisis in the early life history of each unit being balanced by the increasing rate for cancer in the later years.
>
> C. (1) Analysis of the deaths of men in various occupations shows that high phthisis in early life is followed by high cancer among the survivors and low phthisis followed by low cancer.
>
> " Summing up all these points :—
>
> A. Cancer is the specific successor of phthisis.
>
> B. Decrease of phthisis in early life is followed by a specific (because of A) increase of cancer in later life.
>
> C. Phthisis is the index of cancer—opportunity for phthisis means opportunity for cancer."

These views do not appear to have stimulated much research since Cruikshank's very thorough papers of 1939.

That there is a relationship between the two diseases receives further support from a study of the mortality figures since 1939.

1. If the sum of the mortality rates be plotted (combined mortality rate—Fig. 2), then for the past forty years at least, apart from war, these two diseases have taken together an unchanging toll of lives—2500 in 1910, 2300 in 1920, 2500 in 1930, 2500 in 1950, and 2300 in 1953. It is true that peaks of 3000 for males were reached during both wars, but had these emergencies not occurred it is not unreasonable to suppose that the combined mortality curve for males would appear almost as a straight line, as it does in women (Fig. 3), in whom, for an

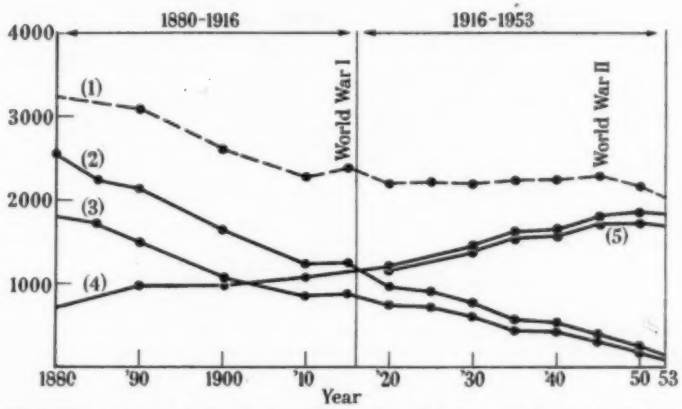

FIG. 3.—Mortality rates per million living, for females, showing combined rate (1), all forms of tuberculosis (2), pulmonary tuberculosis (3), all forms of cancer (4) and non-respiratory cancer (5) from 1880 to 1953.

unknown reason, wartime stress does not appear to increase mortality rates either from tuberculosis or cancer to a comparable degree. This flatness of the combined mortality graph emphasises the exactness by which the mortality rate from tuberculosis has been replaced by that from cancer.

2. Further problems are raised by splitting off from the tuberculosis curve the death rate due to pulmonary tuberculosis, thus differentiating between pulmonary and extra-pulmonary disease and similarly plotting separately the mortality rates for non-respiratory cancer (Fig. 2).

In 1893 the ratio of the mortality rate of pulmonary tuberculosis to extra-pulmonary tuberculosis, i.e. the Tuberculosis Mortality Ratio (T.M.R.) was 2·2. In 1953 the ratio of the mortality rate of non-respiratory cancer to diseases registered under Respiratory Cancer in the Registrar General's returns, i.e. the Cancer Mortality Ratio (C.M.R.) was 2·3.

When these graphs are added a symmetrical figure emerges (Fig. 2), and the fact of the mathematical nature of this diagram has to be explained. The inference would appear to be that there is a relationship between tuberculosis and cancer and also between extra-pulmonary disease and lung cancer,

For purposes of discussion, if it be accepted that there is such a relationship, then by studying the trends of the former condition, light might be thrown on the latter. Reference to Fig. 2 shows that the major cause of the peak on the combined mortality curve for the 1914–1918 war was caused by tuberculosis, the contribution to it from cancer was very small. For World War II, the reverse obtained, the major contribution was due to cancer—the minor one was caused by tuberculosis. Fig. 4 shows the analysis of the tuberculous elements in the

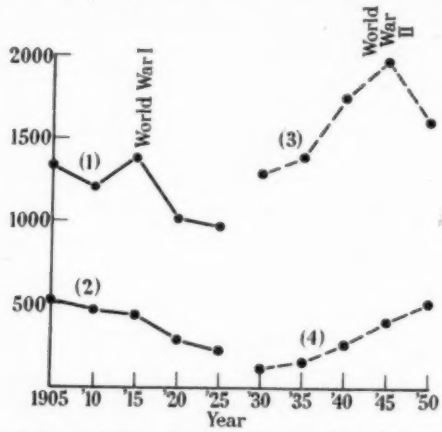

FIG. 4.—Analysis of peaks in mortality rates caused by war for males. (1) Pulmonary tuberculosis. (2) Extra-pulmonary tuberculosis. (3) Non-respiratory cancer. (4) Respiratory cancer.

first war peak and the malignant elements in the second. It will be observed that whereas most of the contribution to the peak in 1914–1918 came from pulmonary tuberculosis, in 1939–1945 it was derived from non-respiratory cancer. In other words extra-pulmonary tuberculosis and lung cancer are apparently only slightly affected by war—a small point in favour of their relationship.

Today, most tuberculous deaths are, in England, due to pulmonary disease. Before 1915 this was not so. If the T.M.R. be calculated from 1890 onwards, it remains around 2·3 until 1915, and then starts to rise until in 1953 it was 10·7 (Table III, Fig. 5). Similarly the C.M.R. had changed in the reverse way—15 in 1920, and 2·3 in 1953. Unfortunately it is not known what the C.M.R. was before 1920, as that was the first year when respiratory cancer was shown separately in the Registrar General's review.

Calculation of these ratios in different areas gives the following results (Table IV, Fig. 6). The T.M.R. is lowest in the country and progressively rises in towns of increasing size and is highest in the conurbations. With the C.M.R. the reverse obtains; it is lowest in the conurbations and highest in the rural areas—in other words the ratio of lung cancer mortality to that of other cancers is greater in big towns, than in the country, and the commonly accepted reason for this is that the larger the town, the higher the level of atmospheric carcinogens, and the greater the incidence of lung cancer. But this does not explain the simultaneous and progressive increase of the T.M.R. in towns of increasing size.

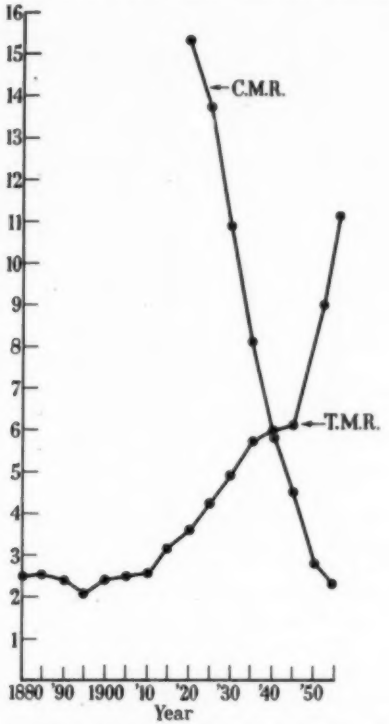

FIG. 5.—The C.M.R. and T.M.R. from 1880 onwards.

$$\text{C.M.R.} = \frac{\text{Non-respiratory cancer mortality rate}}{\text{Respiratory cancer mortality rate}}$$

$$\text{T.M.R.} = \frac{\text{Pulmonary tuberculosis mortality rate}}{\text{Extra pulmonary tuberculosis mortality rate}}$$

TABLE III.—*The Tuberculosis Mortality Ratio and the Cancer Mortality Ratio from* 1880 *onwards*

Year.	Tuberculosis mortality ratio.		Cancer mortality ratio.	
	Males.	Females.	Males.	Females.
1880	2·5	2·4	—	—
1885	2·6	2·4	—	—
1890	2·4	2·4	—	—
1895	2·1	2·1	—	—
1900	2·5	2·1	—	—
1905	2·6	2·1	—	—
1910	2·6	2·2	—	—
1915	3·2	2·8	—	—
1920	3·5	3·2	15·3	50
1925	4·2	3·8	13·6	42
1930	4·9	4·3	11·0	33
1935	5·7	4·9	8·2	32
1940	6·0	4·6	5·9	25
1945	6·0	4·3	4·6	23
1950	8·9	5·7	2·9	18
1953	10·7	5·4	2·3	16·5

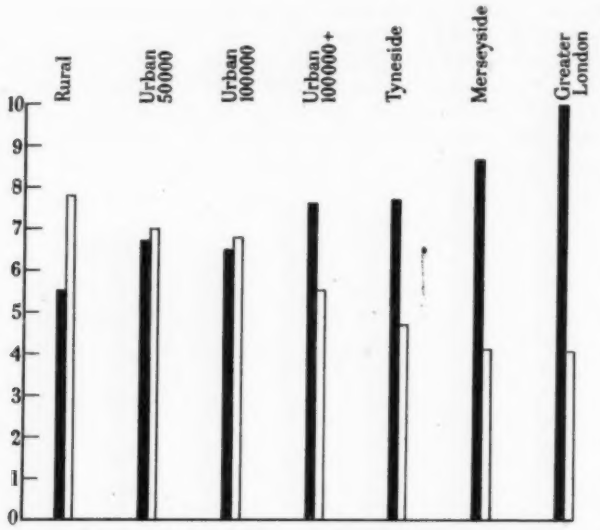

FIG. 6.—The T.M.R. and C.M.R. for rural areas and towns of increasing size.
■ T.M.R. □ C.M.R.

TABLE IV.—*The Tuberculosis Mortality Ratio and Cancer Mortality Ratio for Various Districts during* 1952

District.	Tuberculosis mortality ratio. M.	F.	P.	Cancer mortality ratio. M.	F.	P.
Greater London	13·4	5·9	9·8	2·1	13·0	4·0
Tyneside	11·0	6·9	9·2	2·5	15·5	4·7
Urban England	7·6	4·7	6·7	2·8	18·0	5·3
Dublin	7·0	3·4	5·2	3·2	16·0	5·8
Rural England	6·0	4·2	5·1	4·5	22·4	7·2
Urban Eire	4·9	3·3	4·2	4·2	22·0	7·3
Rural Eire	3·9	3·0	3·4	14·0	26·0	17·0

Is it possible to find a factor the increasing influence of which has been felt since about 1920, and which might conceivably have influenced both conditions simultaneously ?

In the early years of this century, the bovine bacillus was responsible for much of the mortality from extra-pulmonary disease : although it is difficult to ascertain precisely what the actual figure was. Infection with the bovine bacillus was commonly the result of drinking infected milk. From 1920 onwards, three important changes have occurred together ;

(1) Progressive increase in the supply of uninfected milk to the consumer ;
(2) Progressive decrease of extra-pulmonary tuberculosis mortality ;
(3) Progressive increase in lung cancer mortality.

Whether or not this association is accidental, it is a fact that in the rural areas the three changes are least obvious and they are most apparent in the massive towns.

Although there appears to be some relationship in women between tuberculosis and cancer, especially marked over the last forty years there is not the same close correlation between extra-pulmonary disease and lung cancer as in men. In this connection a study of the T.M.R. and C.M.R. for both sexes (Table III) is illuminating. The C.MR. for males shows a change from 15·3 in 1920 to 2·3 in 1953. That for females is less marked—50 in 1920 to 16·5 in 1953. The change in the T.M.R. for women is also less noticeable. It was 3·2 in 1920 and only 5·4 in 1953, whereas the figures for men were 3·5 in 1920 and 10·7 in 1953. Therefore if the decrease in the mortality rate of extra-pulmonary disease is reflected in the increase of the mortality rate of lung cancer, it would not be expected to be so marked in women as in men. This is borne out by the facts.

Cruikshank postulated in 1939 that "There are within the latent tubercle bacilli carcinogenic phages. As long as this 'complex' of bacillus and phages remains intact no disease results, the complex being non-pathogenic. But if this complex breaks down either cancer *or* tuberculosis results." It is here submitted as an extension or ceróllary to this theory that the bovine bacillus plays a major role in the carriage of the phage of lung cancer.

Further evidence in support of a possible relationship between tuberculosis and lung cancer may be obtained by a study of the international figures.

If graphs of the mortality rates for all forms of tuberculosis and lung cancer are prepared for the United Kingdom, the United States and Denmark, it will be found that in the three countries involving a total population of over 150 million white males, the tuberculosis mortality curves are due to strike the zero line about 1960 : also the year 1949 was critical, because in the three countries, in spite of marked differences in the rates, the mortality rate from tuberculosis approximately equalled that from respiratory cancer.

The figures were ;

Males, 1949.	Tuberculosis, all forms.	Respiratory cancer.
United Kingdom . .	530 . .	500
United States . . .	250 .	225
Denmark	160 .	160

Fig. 7 shows, again in the three countries, the rates of tuberculous disease, respiratory cancer and cigarette consumption in 1940 and 1950. It suggests that when international comparisons are made, the higher the tuberculosis mortality rate the higher the respiratory cancer rate, and where the tuberculosis mortality rate is low, e.g. in Denmark, there the respiratory cancer rate is low.

From about 1940 onwards, cigarette consumption in the United States began to outstrip that in Britain. It might be expected that the rate of increase of lung cancer would, as a result, be at least as great by 1953 in the United States as in Britain, although 13 years is but a short time for smoking to act as a causal agent. Reference to Table V shows that this was not so and the rate of increase was actually greater in Britain than in the United States in the proportion of 2·27 to 2·08. It is interesting to observe that during the same period, the rate of decrease of tuberculous mortality was slower in Britain than America in the proportion of 2·07 to 1·85.

Summarising what has here been discussed, further support is given by recent trends of male death rates to the theories propounded by Cherry (1924, 1925, 1933) and Cruikshank (1939) that in a masculine population, infection with the tubercle bacillus in some way predisposes to the development of cancer, so that decrease of the mortality rate of tuberculosis is matched by increase of the mortality

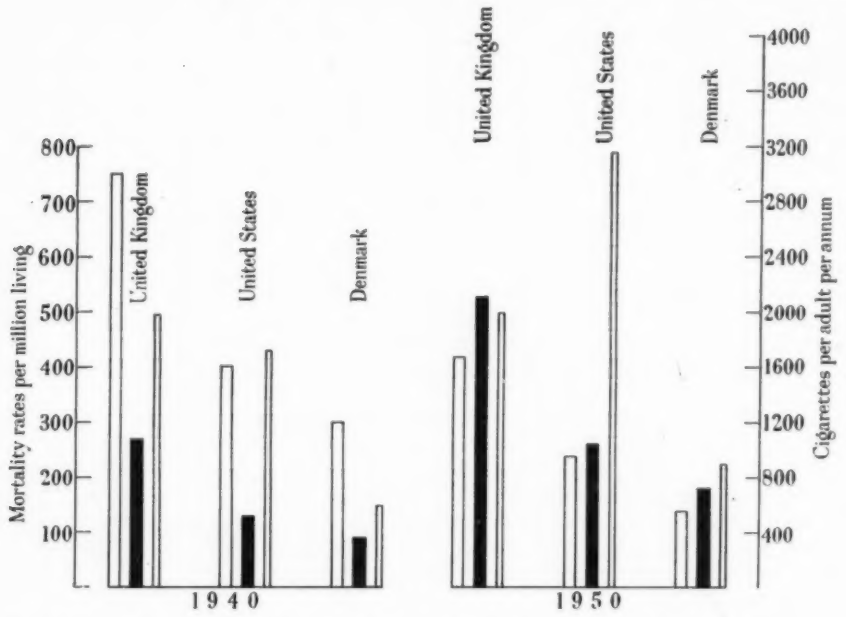

FIG. 7.—Mortality rates per million living for males for all forms of tuberculosis and respiratory cancer, also the cigarette consumption per adult in the United Kingdom, the United States and Denmark during 1940 and 1950. ☐ Tuberculosis. ■ Respiratory cancer. ═ Cigarettes.

TABLE V.—*Ratios of the Mortality Rates in the United Kingdom and the United States of Respiratory Cancer and Tuberculosis and of Cigarette Consumption*

Year.	Respiratory cancer.	Tuberculosis.	Cigarettes.
1940	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{270}{130} = 2{\cdot}08$	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{750}{405} = 1{\cdot}85$	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{1955}{1750} = 1{\cdot}1$
1950	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{530}{260} = 2{\cdot}04$	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{420}{240} = 1{\cdot}75$	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{2029}{3188} = 0{\cdot}6$
1953	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{650}{290} = 2{\cdot}27$	$\frac{\text{U.K.}}{\text{U.S.}} = \frac{280}{135} = 2{\cdot}07$	—

rate from cancer. Artificial lowering of the mortality rate from extra-pulmonary tuberculosis is followed by an increase of the mortality rate from lung cancer. Only in so far as a population has been "predisposed" by a certain level of

tuberculous infection, expressed in terms of the mortality rate it now produces, will tobacco and other smoke produce their carcinogenic effects.

It is fully realised that these views are based on correlations which may be entirely fortuitous, but they are advanced as a possible explanation of the phenomena discussed.

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DESACETYLMETHYLCOLCHICINE IN THE TREATMENT OF MYELOID LEUKAEMIA

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"COLCEMID" (desacetylmethylcolchicine) has been reported as a useful drug in the treatment of chronic myeloid leukaemia, without any serious side effects (Moeschlin, Meyer and Lichtman, 1953; Leonard and Wilkinson, 1955). Our experience has been limited to the treatment of four patients. The results in this small series appear to confirm that "colcemid" will control the disease successfully for a variable period of time in some, but not all, patients, but we do not consider that the toxic effects of this drug can be dismissed as unimportant.

CASE HISTORIES

Case 1

A woman aged 58 was found to have chronic myeloid leukaemia in June 1953. Examination of the blood at that time showed a total white cell count of 500,000 per cu. mm., 87 per cent neutrophils and myeloid precursors. The disease responded to splenic irradiation. Further courses were given with gradually diminishing remissions until July 1955. She was then admitted to hospital as there had been a recent deterioration in her condition. The liver and spleen were enlarged down to the level of the umbilicus and in addition she had developed a generalised adenopathy. Blood examination showed a total white cell count of 57,000 per cu. mm., 67 per cent neutrophils, 12 per cent myeloid precursors; haemoglobin 40 per cent. She failed to respond to blood transfusions, cortisone, 6-mercaptopurine and "colcemid" and died two months after admission.

Case 2

A man aged 60 was found to have polycythaemia in 1947. He received treatment with deep X-rays to the skeleton and later with P.32. The total white cell count was always high and by May 1955 it had risen to 62,000 per cu. mm., 92 per cent neutrophils, 5 per cent myeloid precursors. He was also having a considerable amount of pain from the very marked splenic enlargement. Bone marrow aspiration showed a generalised hyperplasia of all elements. He was given a course of deep X-rays to the spleen, which became impalpable, the white count falling to 40,000 per cu. mm. Six months later the spleen tip could be felt level with the umbilicus and he again complained of pain. The white cell count was 78,000 per cu. mm. but the haemoglobin remained at 108 per cent. The spleen was treated by irradiation three times in the next 18 months with a decreasing response on each occasion. The highest total white cell count recorded

during this period was 126,000 per cu. mm. In November 1955 the patient strained his shoulder while gardening and was admitted two days later as an emergency with an extensive bruise over the right side of the chest. He was also complaining of severe pain from the enlarged spleen which was now palpable in the left iliac fossa. Investigation of the blood showed a haemoglobin of 60 per cent; total white blood cells 146,000 per cu. mm., 83 per cent neutrophils, 10 per cent neutrophil precursors; platelet count 525,000 per cu. mm. He was treated with "colcemid" with an immediate fall in the white cell count and a rise in the haemoglobin, with some diminution in the size of the spleen. The white count rose rapidly when the daily dose of "colcemid" was reduced from

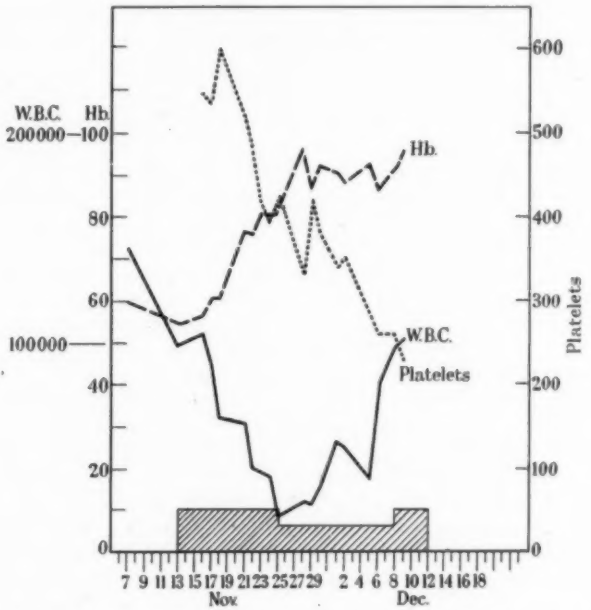

FIG. 1.—Case 2: Effect of "colcemid" on total white cells. Rapid response on reducing daily dose from 5 mg. to 3 mg.

5 mg. to 3 mg. (Fig. 1). The patient died of hypostatic pneumonia before any further observations could be made.

Case 3

A 30-year-old woman was investigated elsewhere in February 1952 for abdominal discomfort of six months' duration. She was found to have chronic myeloid leukaemia with a total white blood cell count of 223,000 per cu. mm. and a haemoglobin of 68 per cent. A satisfactory remission followed a course of deep X-ray therapy to the spleen. Thirteen months later the white cell count had risen to 131,000 per cu. mm. and it again fell rapidly with irradiation. Between

January 1954 and March 1955, three courses of deep X-ray therapy were necessary to control her symptoms and blood count. Each remission was of progressively shorter duration, and it was decided to try the effect of myleran. For 30 days 4 mg. daily were given with some effect on the blood count. The patient was then found to be pregnant. It was thought that this might lead to a deterioration of her leukaemia and, as she already had two children, a hysterotomy was combined with sterilisation. Three months later the total white cell count had risen to 140,000 per cu. mm. and she was again having pain in the left subcostal region. Deep X-ray therapy to the spleen produced a rapid response but the remission was short, and three months later the total white cell count had

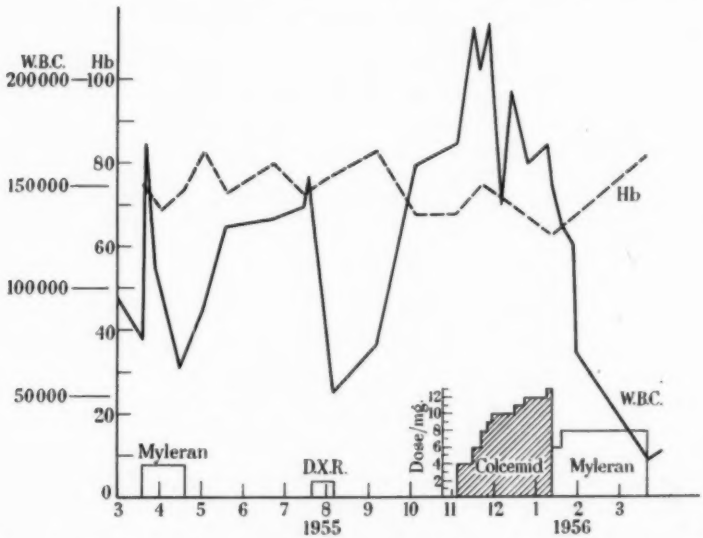

FIG. 2.—Case 3: Resistance to "colcemid" in case responding to X-ray therapy and myleran.

increased to 170,000 per cu. mm. She felt very ill, had lost weight and was again complaining of pain in the splenic region. Treatment with "colcemid" was started with 2 mg. twice daily by mouth. The dose was gradually increased, without any improvement, until a daily total of 10 mg. was reached. At this level, the white cell count showed a slight response, but unfortunately this was not maintaned. The daily dose was then gradually raised to 13 mg. without any benefit. The total white cell count showed no real decrease, the haemoglobin level did not rise appreciably and the spleen remained unaltered in size. The patient continued to feel lethargic and generally unwell, and she became extremely depressed, worrying a great deal over her failure to respond to treatment. It was decided to give myleran a further trial, using a larger initial dose than 4 mg. daily, as this had not proved very effective previously. A satisfactory remission occurred and the total white cell count fell rapidly with a simultaneous rise in the haemoglobin (Fig. 2).

Case 4

A man aged 37 was admitted to hospital as an emergency in September 1955 with severe pain in the left upper abdomen and shoulder, which had been present for three days. He was found to have a firm tender spleen, enlarged to just above the umbilicus. Examination of the blood showed a haemoglobin of 67 per cent and a total white cell count of 148,000 per cu. mm. with a differential count typical of chronic myeloid leukaemia, and the diagnosis was confirmed by bone marrow aspiration. Deep X-ray therapy was given to the spleen, which became impalpable, and the blood count responded satisfactorily. Five months later the total white blood count had increased to 142,000 per cu. mm. and the haemoglobin had fallen. At his next hospital attendance four weeks later he complained once more of left-sided pain and extreme lethargy. It was decided to try the effect of " colcemid ". A satisfactory initial response occurred, and this was maintained on 6 mg. daily in divided doses. The spleen, which had again enlarged to the level of the umbilicus, became painless, but there was no decrease in size during the treatment. His appetite and weight improved and he felt increasingly fit. Unfortunately, the platelet count fell slowly to 90,000 per cu. mm., and the drug was stopped after a symptom-free remission of five months.

Two weeks later, the total white count had risen to 110,000 and the haemoglobin was falling. The patient felt unwell and the original pain returned. Further irradiation was given to the spleen with an immediate fall in the total white cell count and a simultaneous subjective improvement. The spleen shrank a little but the response was disappointing. This remission lasted only two months. The platelet count had by now risen to over 200,000 per cu. mm. and it was decided to try " colcemid " once more. The total white cell count fell rapidly but there was no increase in the haemoglobin level. The drug was stopped for 12 days over Christmas and was recommenced on December 29. Two days later the patient felt extremely ill and his hair suddenly began to fall out. Three days after this he attended for follow-up examination. " Colcemid "was stopped and he was admitted to hospital. There was by this time a severe generalised loss of scalp hair and quantities of hair could easily be removed by gentle combing. He had also noticed thinning of the hair over the rest of the body. His general condition was poor, with pitting oedema of both ankles. The spleen was palpable below the level of the umbilicus and the liver was enlarged to just above this level.

The following relevant investigations were carried out. Blood count: Hb 30 per cent. W.B.C. 107,000 per cu. mm. Neutrophils 17 per cent. Lymphocytes 6 per cent. Monocytes 2 per cent. Eosinophils 1 per cent. Basophils 2 per cent. Metamyelocytes 2 per cent. Myelocytes 43 per cent. Premyelocytes 2 per cent. Myeloblasts 25 per cent. 38 nucleated red cells/100 white cells. Platelets 77,000 per cu. mm. Bone marrow aspiration: differential count identical with peripheral blood. Bone marrow biopsy: Acellular marrow with some increase of fibrous tissue. Serum bilirubin: 1·1 mg. per cent. Reticulocytes: 1 per cent. Coombes test: negative.

A diagnosis of generalised marrow aplasia was made, and further treatment with any cytotoxic drug was thought to be contra-indicated. Repeated blood transfusions, prednisone and cortisone were given to try to postpone the inevitable outcome. Small doses of X-rays were given to the spleen in an attempt to reduce its size and relieve the patient's discomfort but none of these measures appeared

to affect his rapid downhill progress. Terminally the total white cell count rose to over 400,000 per cu. mm., 67 per cent of which were myeloid precursors, and he finally died six weeks after admission to hospital (Fig. 3).

Four days before his death he complained of variable left-sided lower abdominal pain, radiating down the left leg above the knee, and an area of anaesthesia to pin prick and light touch was found over the anterior surface of the affected thigh. The spleen had by then enlarged until it completely filled the

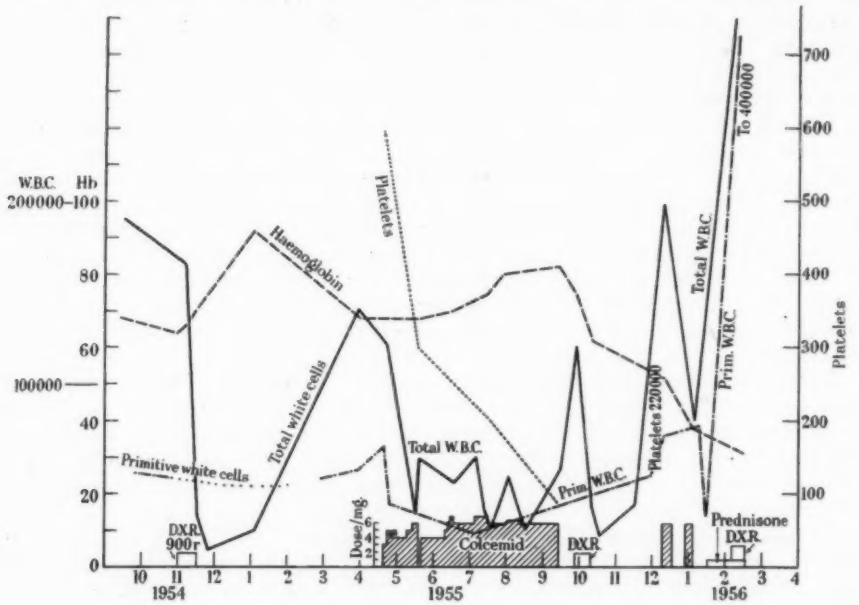

FIG. 3.—Case 4: Control by "colcemid", followed by rapid relapse.

left iliac fossa, and these findings were thought to be due to nerve pressure or actual invasion by the leukaemic process. The autopsy proved these assumptions to be incorrect. During the first two weeks of his stay in hospital, depilation continued at a gradually diminishing rate and over the last week of his life it was thought that there had been some slight regrowth of hair.

Post-mortem examination showed that the spleen was grossly enlarged, weighing 7 lb., and its surface was mottled by old and recent infarcts. The liver weighed 8 lb. and was pale, the macroscopical appearance suggesting myeloid leukaemia. The bone marrow in all the bones examined was pale. On the left lateral and postero-lateral wall of the abdominal cavity, there was a large smooth swelling, pushing the spleen down and the kidney upwards. When the swelling was incised about 2½ pints of semi-solid blood clot was evacuated.

On microscopical examination, the typical changes of myeloid leukaemia were found in the liver, spleen and kidneys. The marrow from all areas was highly cellular. Megakaryocytes and red blood cells were completely absent, and the

cells consisted almost entirely of primitive white cells. The testicles showed a marked maturation defect and skin from both the scalp and the scrotum showed a number of normal hair follicles.

DISCUSSION

Case 1 did not respond to " colcemid " or to any form of treatment. This is not surprising, in view of the advanced stage of the disease.

Case 2 showed a marked fall in the white cell count, associated with a rapidly rising haemoglobin, and some decrease in the size of the spleen. Reduction of the dose of " colcemid " led to an immediate rise in the white cell count. The unexpected death of the patient from bronchopneumonia unfortunately prevented any further observations. This complication cannot be attributed to the drug.

Case 3 proved resistant to " colcemid " in doses of up to a maximum of 13 mg. daily. Leonard and Wilkinson (1955) refer to two out of a total of eight patients with chronic myeloid leukaemia who were resistant to " colcemid " up to a maximum dose of 10 mg. daily. Both their cases were also resistant to myleran and 6-mercaptopurine. No mention is made if they responded to radiotherapy, either before or after " colcemid " therapy. Our case was resistant to " colcemid " but responded to splenic irradiation and to myleran. Resistance to " colcemid " does not therefore mean that other methods of treatment will be unsuccessful.

Case 4 developed loss of scalp hair, which was referred to by Leonard and Wilkinson (1955) as a complication in one of their cases. Their patient also noticed some dysphagia and scrotal tenderness, and the hair loss occurred at the height of the depression of the white cells. No mention is made of regrowth of the hair, and treatment with " colcemid " was abandoned. Our patient developed severe generalised alopecia of the whole scalp and loss of hair in the axillae and on the eyebrows and scrotum. It occurred during the second course of " colcemid " and we are unable to relate this directly to the degree of depression of the white cell count. Although the count at this time was low, the fall was not as marked as it had been on previous occasions during the first course of treatment. No dysphagia or scrotal irritation was noticed and there was no eosinophilia.

This complication has been noted previously during the treatment of human cancer with large doses of colchicine (Seed, Slaughter and Limarzi, 1940). A woman aged 55 with advanced carcinoma of the breast was given intramuscular injections of the drug with some temporary improvement in the tumour. On the 21st day of the treatment her scalp hair suddenly started to fall out in handfuls and in four days she was totally bald. She continued to have injections of colchicine for a further six weeks and, in spite of this, complete regrowth of the scalp hair occurred in five months. If the alopecia produced by " colcemid " is similar, regrowth of the hair may be expected and this was suggested by the findings in our case. It is possible that regrowth of the hair might occur during the continued administration of " colcemid ", but it would require a certain amount of courage to test out this hypothesis.

Before the death of the patient it was thought that marrow failure had developed as a result of treatment, and for this reason no further chemotherapy was given. Although no case of marrow aplasia following the use of " colcemid " had been reported previously, this complication was attributed to the drug

rather than to the natural progress of the disease. The alternative diagnosis of myelosclerosis was considered unlikely as the original bone marrow examination had shown the typical findings of chronic myeloid leukaemia. Some authors believe that myeloid leukaemia and myelosclerosis are one and the same disease (Heller, Lewisohn and Palin, 1947). In fact very few untreated cases have been recorded where the original bone marrow examination showed frank myeloid leukaemia—to be followed at a later date by the development of myelosclerosis.

Post-mortem examination showed that failure of the marrow was due to extensive invasion by primitive forms of the myeloid series. All marrow specimens examined showed these changes, emphasising the danger of relying on the examination of one sample of bone marrow. In light of this knowledge, the response of the leukaemia to the treatment was reviewed and it appeared that " colcemid " was in fact controlling the disease. When the drug was finally stopped, the total count rapidly rose and large numbers of primitive cells of the myeloid series appeared in the peripheral circulation. An alternative explanation that might be offered for the final exacerbation is that the " colcemid " stimulated the leukaemia into an acute phase. Similar charges have been made against deep X-ray therapy, and irradiation is still sometimes blamed when chronic leukaemia enters a terminal acute phase or when the changes of lymphatic leukaemia become manifest in the course of progress of a case of lymphosarcoma. There is very little knowledge of the natural history of untreated leukaemia and without control series of cases, such criticisms must be without foundation.

The chemotherapeutic control of chronic myeloid leukaemia has been achieved for long periods of time without serious complications (Galton and Till, 1955). When chemotherapeutic agents are used it is important that they should be as free from unpleasant side effects, as effective and as easily administered as X-ray therapy. From the small amount of evidence available, we do not consider that " colcemid " is as free from distressing side effects or more effective than X-ray therapy or myleran. We do not think it should be used in the treatment of chronic myeloid leukaemia unless the patient has become refractory both to irradiation and to myleran. Leonard and Wilkinson (1955) refer to the dangers of myleran but it would appear that these have been almost entirely eliminated by the adoption of more suitable dosage schedules (Galton and Till, 1955). " Colcemid " seems to be contra-indicated particularly in the treatment of women, but it might be used with less anxiety if the patient is a man who is already bald. The drug is effective in controlling some cases of chronic myeloid leukaemia and so far there is no evidence that serious marrow depression follows its use.

SUMMARY

An account is given of the treatment of myeloid leukaemia with desacetylmethylcolchicine. The drug appears to be an additional effective weapon in the control of some patients with this disease.

There has been as yet no recorded case of dangerous bone marrow depression during " colcemid " administration, and the only serious toxic effect encountered has been depilation, which is probably reversible.

Depilation is a distressing complication and we do not think " colcemid " should be used in the treatment of chronic myeloid leukaemia, unless the patient no longer responds to splenic irradiation and to myleran.

We wish to thank Professor B. W. Windeyer and Miss M. D. Snelling for allowing us to report patients under their care. Part of the expenses of this investigation has been defrayed by the British Empire Cancer Campaign.

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POLYCYCLIC HYDROCARBONS IN CIGARETTE SMOKE: THE AMOUNTS HELD IN STUBS AND ASH

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In earlier publications (Cooper, Lindsey and Waller, 1954; Cooper and Lindsey, 1953, 1954, 1955), the amounts of a number of polycyclic hydrocarbons present in mainstream cigarette smoke produced by a method resembling human smoking were determined and, by smoking "cigarettes" prepared entirely from paper, an estimate was made of the amounts produced by the paper of a normal cigarette (Cooper, Gilbert and Lindsey, 1955). The present investigation is an attempt to determine the total amounts of these compounds produced by finding the amounts retained in the stubs and ash of cigarettes, and proves that cigarette tobacco smoked in a pipe gives rise to the same hydrocarbons although the conditions of smoking are different.

EXPERIMENTAL

The glass apparatus was cleaned and all solvents and reagents were purified as described previously (Commins, Cooper and Lindsey, 1954). Smoking experiments were carried out with the machine formerly used and the conditions of smoking were as then described.

Examination of stubs

Stubs, of average length 1·5 cm., dried *in vacuo* over sulphuric acid, were extracted to exhaustion with cyclohexane in a Soxhlet extractor and the resulting solution washed three times in turn with 2N sulphuric acid, water, 2N sodium hydroxide, and water. The hydrocarbons were then determined in the neutral solution by the method previously described. The quantities of the various hydrocarbons from 500 cigarette stubs are listed in Table I.

TABLE I.—*Polycyclic Hydrocarbons in Stubs from 500 Cigarettes in Micrograms*

Acenaphthene	31·5
Acenaphthylene	18·8
Fluorene	39·6
Phenanthrene	37·5
Anthracene	58·3
Pyrene	26·7
Fluoranthene	181·0
1 : 2-Benzanthracene	42·1
Perylene	1·6
3 : 4-Benzpyrene	9.8
Anthanthrene	1·4
Coronene	54·8

Naphthalene and 2-methylnaphthalene were also detected.

Examination of ash

The ash from cigarettes smoked mechanically in the standardized manner was collected, dried *in vacuo* over sulphuric acid and extracted to exhaustion in a Soxhlet extractor with cyclohexane. The extract was analysed in the manner previously described and the quantities found are tabulated in Table II. Approximately 38 g. of ash are produced from 500 cigarettes and the amounts are computed for this figure.

TABLE II.—*Polycyclic Hydrocarbons in Ash from 500 Cigarettes in Micrograms*

Acenaphthene	3·9
Acenaphthylene	0·8
Fluorene	1·7
Phenanthrene	0·08
Anthracene	0·8
Pyrene	0·5
Fluoranthene	0·4
1 : 2-Benzanthracene	0·5
3 : 4-Benzpyrene	0·3
Coronene	0·1

Azulene and 2-methylnaphthalene were also detected.

Examination of cigarette tobacco

The tobacco was obtained by opening cigarettes of the same type as were used in the previous experiments; it was then smoked in a pipe of pyrex glass attached to the smoking machine. The conditions of smoking were made to conform more closely to those of pipe smoking than to those of cigarette smoking; that is by using more frequent and shorter puffs until only a small "dottle" of tobacco remained. The total smoking time was considerably longer than was necessary with the same weight of cigarettes. The smoke was collected in cyclohexane flasks and the long glass precipitator previously employed and the analysis carried out exactly as described for cigarette smoke. The quantities of various hydrocarbons from 440 g. of tobacco are listed in the first column of Table III (this quantity was chosen because it is the amount consumed when 500 cigarettes are smoked in the standardized manner).

TABLE III.—*Polycyclic Hydrocarbons from Cigarette Tobacco and Paper*

(Amounts in Micrograms)

	From 440 g. tobacco	From 16·7 g. paper	Total
Acenaphthylene	132·0	1·4	133·4
Anthracene	104·0	0·7	104·7
Pyrene	200·2	2·9	203·1
3 : 4-Benzpyrene	34·9	0·7	35·6

Temperature measurements

Average temperatures in the smouldering "coal" in various smoking experiments have previously been reported (Wynder, Graham and Croninger, 1953; Lindsey, 1954; Doll, 1955). The latest measurements were made with a thermocouple of gold-palladium and platinum-iridium alloys ("Pallador" thermojunction) inserted into the smoking material. The electromotive force produced was measured by means of a precision potentiometer with the cold junction in

melting ice, and the calibration was confirmed by standardizing with pure molten zinc. Surface temperatures were measured with a disappearing filament pyrometer. The results are tabulated below :—

	Quiescent combustion temperature (° C.)	Suction combustion temperature (° C.)	Surface temperature (° C.)
Ordinary cigarette	650	700	900+
Paper cigarette	Varies	655	900+
Pipe	,,	470	700+
Cigar (Havana)	400–500	560	800+

DISCUSSION

The examination of stubs and ash gives information that, with the figures previously reported (Cooper, Gilbert and Lindsey, 1955), provides additional knowledge on the distribution of the combustion products during normal smoking. Thus in Table IV are listed the hydrocarbons found in the smoke from 500 cigarettes and, for comparison, the amounts condensed in the stubs and ash. Four important hydrocarbons are thus studied ; those for which determinations have been repeatedly made in smoke of various kinds.

TABLE IV.—*Polycyclic Hydrocarbons found on Smoking* 500 *Cigarettes*

(Amounts in Micrograms)

	Mainstream smoke	Stubs	Ash	Total
Acenaphthylene	20·5	18·9	0·8	40·2
Anthracene	48·0	59·0	0·8	107·8
Pyrene	55·0	26·7	0·5	82·2
3 : 4-Benzpyrene	4·0	10·0	0·3	14·3

It is noteworthy that, of the total hydrocarbon amounts, a considerable fraction remains in the stubs and further that the proportions of the various hydrocarbons in the stubs are not those found in the mainstream smoke. This could be ascribed to selective absorption in the stubs, but, bearing in mind the physical nature of the smoke—which is an aerosol with the disperse phase consisting of fine viscous droplets—is more probably due to a fractionation process in which the readily aggregated smoke droplets are repeatedly re-volatilized as the smoking proceeds. Thus the material retained in the stubs is richer in the less volatile constituents. It is also likely that, in addition to this redistillation process, there are specific effects due to compounds in the unburnt portion acting as solvents for or combining with the smoke constituents. Simple volatility is certainly not the only effect or more pyrene would be retained in the stubs.

There was no evidence that selective condensation occurred in previous experiments on " Denicotea " filters (Cooper and Lindsey, 1955) where the relative proportions of the hydrocarbons were found to be approximately the same as in the mainstream smoke. Here the temperature remains low and little redistillation would be expected.

The results of the pipe smoking together with those of paper smoking are shown in Table III. In these experiments exactly the same materials were smoked as those described in Table IV but the total amounts of hydrocarbons produced are much greater. The conditions of smoking are very different ; the most

significant being the considerably lower temperature of smoking in the pipe experiments and the longer time of smoking. It has previously been stated that high temperatures favour pyrolysis of organic material to polycyclic hydrocarbons (Kennaway, 1925; Doll, 1955) but some other experiments (Kennaway, 1924) have indicated the reverse tendency. The present and other experiments conducted with pipe smoking mixtures indicate that there is a temperature range within which maximum amounts are formed. Further investigations are being conducted to determine this point. The attempt to confirm the amounts of hydrocarbons produced upon smoking whole cigarettes by smoking tobacco and paper separately have been unsuccessful because it is impossible to smoke cigarette tobacco alone in the same way as it is smoked in a cigarette.

Although several investigators have recently confirmed our finding of polycyclic hydrocarbons in cigarette smoke, Kuratsune, in a very recent paper (1956) reported no 3 : 4-benzpyrene present. He could however, detect it in quantities much smaller than those found in the present investigation in stubs and ash. The conditions employed by Kuratsune were most unlike those occurring in normal human smoking and no mention was made of any other hydrocarbons.

SUMMARY

1. The proportions of polycyclic aromatic hydrocarbons in cigarette smoke, stubs and ash have been determined and an estimate of the total amounts formed during normal smoking has been made.

2. Improvements in chromatographic techniques have made possible the detection of more hydrocarbons in some of the smoke products. A full list of all compounds of this class detected in this and previous investigations is: acenaphthene, acenaphthylene, anthanthrene, anthracene, azulene, 1 : 2-benzanthracene, 3 : 4-benzpyrene, 1 : 12-benzperylene, coronene, fluoranthene, fluorene, 2-methylanthracene, 2-methylnaphthalene, naphthalene, perylene, phenanthrene, 3-methylpyrene, pyrene.

3. Attempts to confirm the amounts of polycyclic aromatic hydrocarbons by smoking cigarette tobacco in pipes were unsuccessful because of the very different conditions. Much greater amounts were found in pipe smoking. Previous conclusions that the tobacco in cigarettes provides the major contribution to the amounts of these compounds are substantiated.

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POLYCYCLIC HYDROCARBONS IN TOBACCO SMOKE: PIPE SMOKING EXPERIMENTS

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PRELIMINARY experiments on pipe smoking have been reported (Cooper, Gilbert and Lindsey, 1955; Gilbert and Lindsey 1956), and it has been shown that with cigarette tobacco smoked in this way the amounts of polycyclic hydrocarbons produced were much larger than when the same amount of tobacco was smoked in the form of cigarettes. The present investigation is of two pipe tobaccos of popular types sold in this country and of herbal smoking mixtures, one blended for pipe smoking and the other for cigarette smoking.

EXPERIMENTAL

Preparation and analysis of smoke

The smoking machine previously described (Cooper and Lindsey, 1955) was used with a pyrex pipe fitted in place of the cigarette holder manifold. Conditions of smoking were adjusted to conform to the more usual behaviour of pipe smokers, that is with shorter puff duration and shorter intervals between puffs than with cigarettes. The tobacco was tamped down from time to time with a glass disc on the end of a rod and, as with cigarette smoking, a lamp burning pure alcohol was used as a lighter. The smoke passed in turn through two flasks of cyclohexane and the long glass precipitator previously described. Precautions regarding cleaning of apparatus and purification of solvents and the methods of separation and analysis were also as described previously.

Considerable difficulty was experienced in separating sufficiently pure chromatographic fractions for accurate determination of the hydrocarbons by spectrophotometry, but by repeated chromatography the general light absorption was reduced sufficiently for characteristic absorption bands to be recognisable and measurable.

Analytical results

The smoking mixtures were a light coarse cut pipe tobacco, a dark brown, highly aromatic pipe tobacco, a coarse herbal mixture for use in pipes and a fine cut herbal mixture for use in cigarettes. The amounts of various hydrocarbons produced from 100 g. of smoking material consumed are shown in Table I.

DISCUSSION

It is well known that a number of dark pipe tobaccos contain fire-cured material (Garner, 1956; Naghski, Beinhart and Couch, 1944). This process involves treating the leaves in the smoke from smouldering damp sawdust for long periods,

TABLE I.—*Polycyclic Hydrocarbons in Pipe Smoking Mixtures*

	Micrograms per 100 grams			
	Light pipe tobacco	Dark pipe tobacco	Herbal pipe mixture	Herbal cigarette mixture
Naphthalene	18·2	28·9	22·3	—
Azulene	5·1	7·0	8·0	4·5
Acenaphthylene	29·1	595·9	87·0	136·0
Fluorene	70·5	1028·0	435·0	613·0
Phenanthrene	296·8	665·0	239·5	104·2
Anthracene	110·0	198·0	103·8	162·0
Pyrene	79·5	122·0	62·9	55·3
Fluoranthene	14·7	167·1	37·1	104·5
3-Methylpyrene	25·0	21·7	14·7	47·1
1 : 2-Benzanthracene	30·8	124·5	36·5	24·4
3 : 4-Benzpyrene	8·5	11·3	8·9	6·5
Anthanthrene	0·5	1·2	0·4	0·3
Coronene	2·1	10·7	2·3	11·4

and it was thought that the results obtained in the analysis of the second sample might be explained if this process had been employed in its manufacture. Extraction of the dried tobacco in a Soxhlet extractor with cyclohexane gave a solution that, when analysed after preliminary acid and alkali washing, showed considerable amounts of polycyclic aromatic hydrocarbons. The presence of these compounds in the unburnt material raises the general question of their probable presence in other unburnt tobaccos, and so a series of investigations was made of all the other smoking materials previously examined. These will be reported in the following publication.

An explanation of the presence of hydrocarbons in unburnt tobacco may be that the leaves in the season of growing take up these compounds as deposits from atmospheric soot. They may also acquire them from atmospheric soot in the various stages of manufacture.

Polycyclic hydrocarbons, if present before smoking, will doubtless be destroyed in part when smoked, but they may also be volatilized and thus find their way into the mainstream smoke. Thus the amounts found in the smoke may not entirely be due to the combustion process. In a recent report (Lyons, 1955) it was shown that 3 : 4-benzpyrene, introduced as a solution into cigarettes was recovered in the mainstream smoke only to 20 per cent of the added amounts when the cigarettes were smoked.

SUMMARY

1. Pipe tobaccos and herbal smoking mixtures, smoked mechanically in a machine simulating human pipe smoking, produce condensates containing much higher proportions of polycyclic aromatic hydrocarbons than were obtained from cigarette smoke.

2. The following compounds were detected and determined, acenaphthylene, anthanthrene, anthracene, azulene, 1 : 2-benzanthracene, 3 : 4-benzpyrene, coronene, fluoranthene, fluorene, 3-methylpyrene, naphthalene, phenanthrene and pyrene.

3. Extraction of the dried tobaccos with cyclohexane showed that the hydrocarbons are present in smaller amount before smoking. This matter is the subject of the investigation reported in the following paper.

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POLYCYCLIC HYDROCARBONS EXTRACTED FROM TOBACCO: THE EFFECT UPON TOTAL QUANTITIES FOUND IN SMOKING

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In a recent investigation, the presence of polycyclic aromatic hydrocarbons in unsmoked Venda tobacco was demonstrated (Campbell and Cooper, 1955) and in consequence of these findings and in connection with other studies concerning the amounts of such compounds in pipe tobacco smoke (Gilbert and Lindsey, 1956) a series of experiments was carried out to determine the amounts in unburnt cigarettes, cigarette paper, pipe tobaccos and herbal smoking mixtures. These investigations are reported below.

EXPERIMENTAL

All the materials examined were dried *in vacuo* over sulphuric acid and extracted to exhaustion with cyclohexane in a Soxhlet extractor. The solutions so formed were treated with acid and alkali and subjected to chromatographic separation and spectrophotometric determination by the method used for cigarette smoke (Cooper and Lindsey, 1955).

The smoking materials so examined were Virginia cigarettes of the type used in previous experiments, paper " cigarettes ", a light pipe tobacco, a dark brown highly aromatic pipe tobacco, a coarse herbal mixture for use in pipes and a fine herbal mixture for use in cigarettes. All these smoking mixtures were identical with those used in recent studies of pipe smoke (Gilbert and Lindsey, 1956). In Table I, column A, are shown the results of these determinations of extractable hydrocarbons.

The extracted materials were re-humidified by keeping them in a closed vessel over water until they had taken up water vapour to about 12 per cent, and they were then smoked in pipes (except for the cigarettes and paper " cigarettes ") as described in previous investigations. The polycyclic hydrocarbons were then determined in the smoke samples. These amounts also are shown in Table I, column B, and for comparison in column C, the amounts of the hydrocarbons previously determined in the smoke of un-extracted smoking materials are shown. All the quantities refer to 100 g. of material extracted or consumed in smoking.

Several interesting points are demonstrated in the table. Firstly it should be noted that pipe smoking of all mixtures, whether extracted or not, gives rise to much greater quantities of hydrocarbons than have been found in cigarette smoke. This point has been noted and discussed in connection with cigarette tobacco smoked in pipes (Gilbert and Lindsey, 1956). Secondly, the presence of poly-

TABLE I.—*Polycyclic Hydrocarbons in Micrograms from* 100 *grams*

	Cigarettes			Paper			Pipe tobacco (light)		
	A	B	C	A	B	C	A	B	C
Acenaphthylene	—	0·3	5·0	—	3·3	8·3	—	2·0	29·1
Phenanthrene	—	13·0	—	—	10·9	4·3	—	80·0	297·0
Anthracene	0·1	2·2	10·9	0·1	4·0	16·9	0·3	58·0	110·0
Pyrene	10·3	3·3	12·5	1·4	9·2	17·0	19·5	40·0	75·5
Fluoranthene	10·6	2·3	—	2·1	8·6	16·9	12·6	15·0	14·7
3 : 4-Benzpyrene	0·25	0·4	0·9	0·1	1·4	4·0	0·5	3·0	8·5

	Pipe tobacco (dark)			Herbal pipe mixture			Herbal cigarette mixture		
	A	B	C	A	B	C	A	B	C
Acenaphthylene	—	8·9	596·0	—	8·2	136·0	—	6·5	87·0
Phenanthrene	—	840·0	665·0	—	180·0	104·2	—	400·0	240·0
Anthracene	—	88·5	198·0	2·0	55·0	162·0	0·5	81·0	7·8
Pyrene	78·0	72·0	122·0	12·0	57·0	55·3	20·5	45·0	62·9
Fluoranthene	77·0	57·0	167·0	15·8	21·0	104·5	21·5	58·0	37·1
3 : 4-Benzpyrene	5·4	7·0	11·3	0·3	13·0	6·5	1·4	6·2	8·9

cyclic hydrocarbons in unsmoked material is demonstrated in all the materials studied. The amounts are usually smaller than those found upon smoking and it must be assumed, as has been demonstrated recently for 3 : 4-benzpyrene (Lyons, 1955), that all the hydrocarbons originally present are partially destroyed on smoking. However, no uniformity is discernible between the quantities removed by extraction and the amounts found upon smoking. This must be attributed to the varied conditions of combustion, especially in pipes, of materials of very variable composition and of different origin. The extraction process removes considerable amounts of substances other than hydrocarbons (e.g. carotene and lycopene) and these and other unknown materials are therefore not available for the smoking process in the extracted materials.

It has been suggested that atmospheric pollution may contribute towards the hydrocarbon content of unburnt tobaccos (Gilbert and Lindsey, 1956) and in order to make an assessment of the contribution from this source, a Virginia-type tobacco grown in Hertfordshire during the season 1954, and air-cured was also examined by the extraction process. The amounts of the various hydrocarbons found are shown in Table II, which also gives similar figures for fresh tobacco leaves flown from Rhodesia in a sealed cannister, and the Venda tobacco experiments described by Campbell and Cooper (1955). It will be noted that the English-grown tobacco upon extraction gives results closely similar to those given by the herbal mixtures which were mostly grown in England (Table I). It is also interesting to note that leaves freshly plucked show very small amounts of hydrocarbons; presumably because there was no opportunity of contamination by smoke in Rhodesia.

TABLE II.—*Polycyclic Hydrocarbons in Micrograms extracted from* 100 *grams.*

	English tobacco (air-cured)	Rhodesian tobacco (fresh leaves)	Venda tobacco
Anthracene	0·2	—	1·0
Pyrene	10·1	1·1	3·0
Fluoranthene	15·2	0·5	11·0
3 : 4-Benzpyrene	0·3	—	0·6

The examination of laurel leaves grown in smoke polluted areas has previously been used to measure atmospheric pollution (Coste, 1945). An independent test of the amounts of the polycyclic hydrocarbons taken up by leaf surfaces in England was therefore made by cutting leaves of the current years growth from the cherry laurel (*Prunus cerasifera*). These were collected on December 8, 1955 after a dry period of several weeks at three places ; one mile from Rickmansworth, Hertfordshire, a similar distance from Sevenoaks, Kent, and in Hyde Park, Central London. The smoke pollution was removed from the leaves by swabbing both sides with hydrocarbon-free cotton wool soaked in chloroform. The swabs were extracted with chloroform to exhaustion in a Soxhlet extractor, the dissolved material transferred to cyclohexane and the hydrocarbons determined by the method of Cooper (1954). The area of the leaves used was determined and in Table III the amounts of hydrocarbons found per unit area is shown.

TABLE III.—*Polycyclic Hydrocarbons Deposited on Cherry Laurel Leaves*

	Micrograms per square metre		
	Sevenoaks	Rickmansworth	Hyde Park
Anthracene	—	—	1·3
Pyrene	2·9	2·4	16·3
Fluoranthene	3·8	3·3	12·2
3 : 4-Benzpyrene	2·8	2·4	13·5
1 : 12-Benzperylene	—	—	4·1

These quantities accord well with the proportions of smoke pollution found at the places named by snow analysis (Gilbert and Lindsey, 1955). No comparison can be made with the tobacco results since the leaf area of the sample grown in England could not be determined. It should be noted that more benzpyrene in proportion to the other hydrocarbons was found in the laurel experiments. Judging from the smoking materials grown in England (tobacco and herbal mixtures), an average of about 30 μg. total measurable hydrocarbons are extractable from 100 g. and an average of less than 1 μg. of this is 3 : 4-benzpyrene. If the results of Lyons (1955) may be applied to these very small amounts (a debatable matter) about 20 per cent of this passes into the mainstream smoke, and compared with the amounts found on smoking, this may be neglected.

The general conclusion from these experiments upon a most varied selection of smoking mixtures is that the smoking process makes the major contribution to the amounts of polycyclic hydrocarbons present in the mainstream smoke.

SUMMARY

1. Polycyclic aromatic hydrocarbons have been extracted with cyclohexane from a number of smoking materials including cigarettes, various tobaccos, herbal smoking mixtures and cigarette paper.

2. The extracted materials, after re-humidifying, gave upon smoking further considerable amounts of hydrocarbons but these amounts were less than the amounts formed upon the smoking of the un-extracted materials. The smoking process produces the major amounts of the hydrocarbons.

3. It is concluded that atmospheric smoke pollution may in part account for the presence of these compounds in the smoking materials. Manufacturing processes may also have an effect, especially the blending of fire-cured tobacco

in some mixtures. Tobacco leaves flown in sealed cannisters direct from a plantation in Rhodesia contained negligible amounts of polycyclic aromatic hydrocarbons.

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THE INDUCTION OF TUMOURS OF THE SUBCUTANEOUS TISSUES, LIVER AND INTESTINE IN THE MOUSE BY CERTAIN DYE-STUFFS AND THEIR INTERMEDIATES

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THE purpose of the experiments about to be described was to test a number of amines and azo-dyes for carcinogenic activity in the mouse, a species which has been comparatively little used for the investigation of this type of compound. The chemicals chosen were 2 dyes (auramine and magenta), the manufacture of which constitutes an industrial hazard according to Case and Pearson (1954); 6 dyes formerly or at present in use as food colorants (carmoisine, rhodamine B, sunset yellow, ponceau 2R, xylylazo-2-naphthol and *o*-tolylazo-2-naphthol); 3 dyestuffs intermediates (benzidine, 1-naphthylamine and 2-naphthylamine); and 2 ortho hydroxy amines related to known metabolites of aromatic amines (3 : 3′-dihydroxybenzidine hydrochloride and 0-methyl-2-amino-1-naphthol hydrochloride).

The tumours induced by the chemicals were of three types: subcutaneous sarcomas, hepatomas and intestinal polyps and carcinomas.

Spontaneous intestinal tumours in the mouse have been described occasionally (Table I). Murray (1905, 1908) found 2 adenocarcinomas in the small intestine invading the wall of the gut and expanding under the peritoneum. The drawings of these tumours clearly indicate their nature. Slye, Holmes and Wells (1917) described a squamous carcinoma of the rectum arising in the prolapsed organ, the only intestinal tumour observed in 16,500 *post-mortem* examinations on mice. Slye (1924) mentioned that 2 squamous carcinomas of the rectum had been seen at *post-mortem* in 40,370 mice and one of the duodenum in 1600 mice. Strong (1941) stated that strain A mice were liable to develop spontaneous tumours of the caecum.

Bonser and Jull (unpublished observation) found two caecal polyps in a female albino stock mouse, bought from a dealer but having no relation to the mice used in the present experiments, and treated for 136 weeks by means of subcutaneous injections of arachis oil. These tumours were composed of irregular intestinal glands, some of which were cystic and dipped through the muscularis mucosae into the subepithelial tissues, there evoking a mild inflammatory reaction. They also observed a caecal carcinoma in a female mouse of the same stock in which a paraffin wax pellet had remained implanted in the bladder for 40 weeks with negative result. The mouse was thus 52–56 weeks of age. This tumour was an anaplastic bowel carcinoma, invading the muscular wall as far as the serosa and permeating subserosal and mesenteric lymphatic vessels. Embedded in the tumour were cystic spaces lined by mucus-secreting columnar epithelium similar to those seen in the polyps, but situated at all levels in the muscular wall and also beneath the serosa. From this appearance it was decided that the tumour arose in a benign polyp.

Tumours have been induced in the mouse intestinal tract by a variety of chemicals (Table II). Badger, Cook, Hewett, Kennaway, Kennaway, Martin and Robinson (1940) described a carcinoma of the small intestine in one of 10 mice receiving 6-methyl-3 : 4-benzphenanthrene by mouth for 394 days. Lorenz and Stewart (1940) described local and metastasising carcinomas of the small intestine in mice of the A strain receiving 1 : 2 : 5 : 6-dibenzanthracene and of the A backcross receiving 20-methylcholanthrene in an olive oil emulsion in the drinking water. Later (1941) these authors found similar tumours in mice of other strains treated with these chemicals, together with one carcinoma of the caecum and a few in the upper part of the colon. White and Stewart (1942) observed small intestinal polyps and carcinomas and also haemangio-endotheliomas in the intestinal wall and other abdominal sites in mice of the C3H and C strains receiving methylcholanthrene in the diet. The photographs and morbid anatomical descriptions of these tumours are extremely lucid. More males than females were treated and only one tumour was stated to have occurred in a female. Foulds (1947) described a small intestinal carcinoma with a secondary deposit in a regional lymph node in one male out of 25 receiving 2-acetylaminofluorene in the diet.

Thus it would appear that spontaneous benign and malignant tumours of the intestine occur but rarely in the mouse. The sex incidence is not known. Induced tumours resemble the spontaneous ones in site and structure and have been more commonly described in males.

Source of mice.—The mice were brought from a local dealer, who has now gone out of business. They were divided into groups of 30, comprising equal numbers of each sex, and were kept 5 in a box. Where the mortality was high in the early weeks of the experiment, another 30 mice were subsequently added. They were fed on rat cake, supplemented by cod liver oil and marmite and were given water *ad lib.* All mice used in the experiment subsequent to April 1953 were vaccinated on the tail with calf lymph as a protection against ectromelia.

Administration of the chemicals.—At first, a standard technique was adopted of subcutaneous injection twice weekly of a solution or suspension of the chemical

EXPLANATION OF PLATES

FIG. 1.—Mouse H 285, male. Forty-six weeks from start of treatment with benzeneazo-2-anthrol. Vasoformative sarcoma or haemangio-endothelioma of back muscles. A fragment of the destroyed vertebral body is seen top middle. × 150.

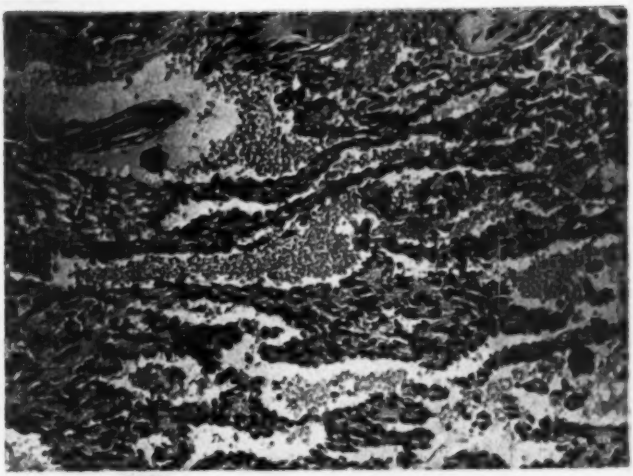
FIG. 2.—Mouse H 127, male. Seventy weeks from start of treatment with arachis oil. Benign early polyp of caecum, showing regular proliferating glands lined by tall epithelium with hyperchromatic nuclei and granular cytoplasm, secreting little mucus, situated superficial to the muscularis mucosae. There is inflammation and fibrosis of the stroma. × 102.

FIG. 3.—Mouse H 69, female. Sixty-five weeks from start of treatment with *o*-tolylazo-2-napthol. Benign polyp of ileum, composed of irregular proliferating glands which are beginning to invade the muscular coat (top centre). There is fibrosis of the stroma. × 40.

FIG. 4.—Mouse H 702, female. Seventy-six weeks from start of treatment with 0-methyl-2-amino-1-naphthol. Benign polyp of caecum, with cystic glands penetrating the muscular coat and provoking a zone of inflammation in the serosa. × 26.

FIG. 5.—Mouse H 379, female. Eighty-four weeks from start of treatment with fresh oily solution of 2-naphthylamine (B.D.H.). Malignant polyp of caecum, composed of irregular glands lined by tall or cubical epithelium, with hyperchromatic nuclei, granular cytoplasm, and little mucus secretion, though some of the glands are cystic. The line of the base is irregular and the muscularis mucosae is partially destroyed. × 102.

FIG. 6.—Mouse H 97, male. Sixty-two weeks from start of treatment with *o*-tolylazo-2-naphthol. Anaplastic carcinoma invading the muscular wall as far as the serosa . Tumour emboli present in subserosal lymphatic vessels. Two cystic glands in muscle resembling those seen in benign polyps. × 30.



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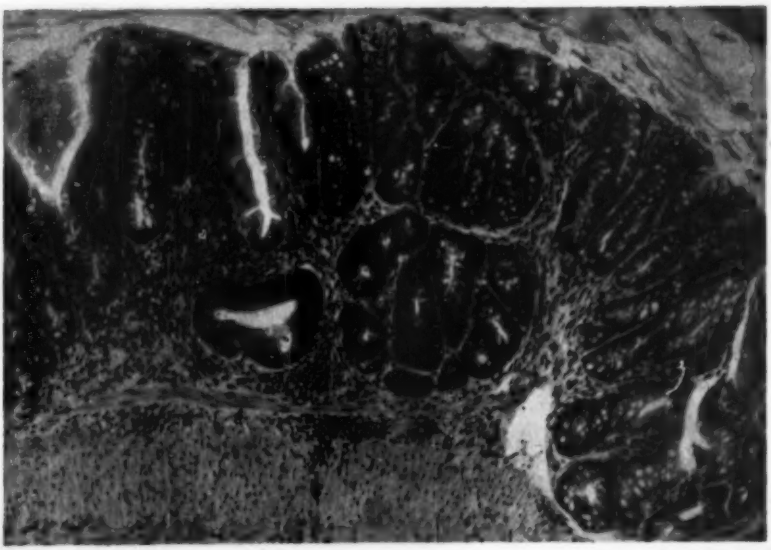

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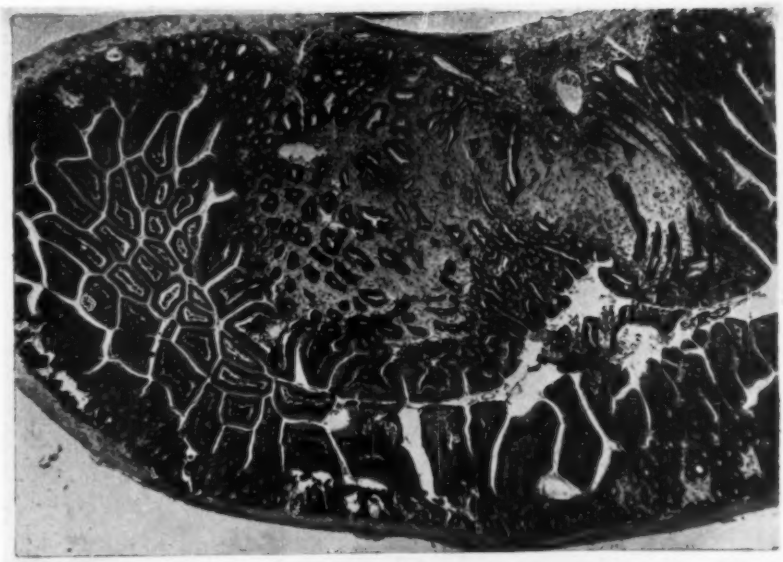

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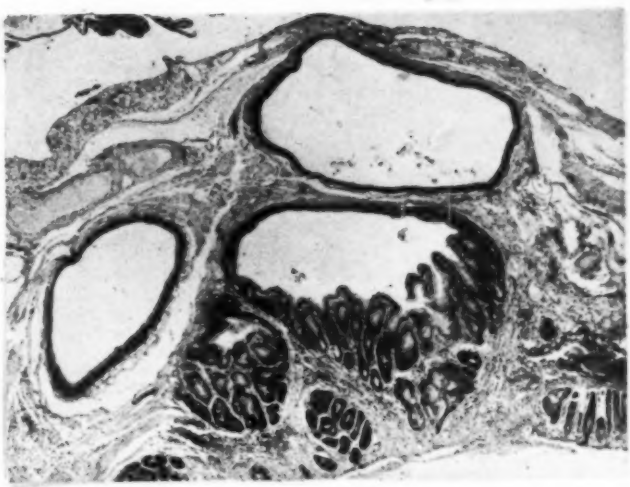

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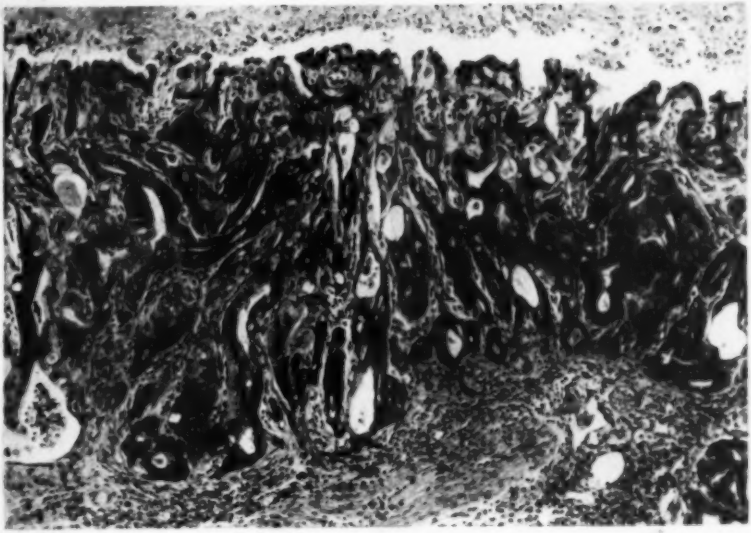

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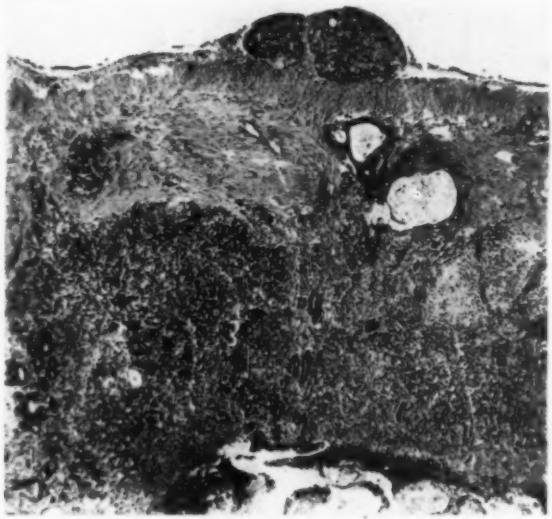


6

TABLE I.—*Spontaneous Tumours of the Small and Large Intestine in the Mouse*

	Small intestine		Large intestine	
	Adenoma	Adenocarcinoma	Adenoma	Adenocarcinoma
Present series	1	1	1	1
Literature	1	1	1	1

TABLE I.—*Spontaneous Tumours of the Small and Large Intestine in the Mouse*

Author	Type of mouse	Age in weeks	Sex	Small intestine: Site	Small intestine: Benign	Small intestine: Malignant	Small intestine: Incidence	Large intestine: Site	Large intestine: Benign	Large intestine: Malignant	Large intestine: Incidence
Murray, 1905	Not stated	Not stated	Not stated	Not stated	—	Adeno carcinoma invading muscular wall	Not stated	—	—	—	—
1908	,,	,,	,,	,,	—	Ditto	,,	—	—	—	—
Slye, Holmes, and Wells, 1917	Not stated	116	Male	,,	—	—	,,	Prolapsed rectum	—	Squamous carcinoma	1/16,500
Slye, 1924	,,	Not stated	Not stated	Duodenum	—	Carcinoma	1/41,165	Rectum	—	Ditto	1/40.370
Bonser and Jull (unpub.)	Stock from breeder, injected with arachis oil	148	Female	—	—	—	—	Caecum (multi focal)	Adenoma with downgrowth of cystic epithelium	—	1/5
	Stock from breeder, bladder implanted with paraffin wax pellet	52–56	,,	—	—	—	—	Caecum (annular)	—	Anaplastic adeno-carcinoma with lymphatic permeation	1/881

TABLE II.—*Induced Tumours of the Small and Large Intestine in the Mouse*

Author	Type of mouse	Age in weeks	Sex	Chemical	Mode of administration	Small intestine: Site	Small intestine: Benign	Small intestine: Malignant	Small intestine: Incidence	Large intestine: Site	Large intestine: Benign	Large intestine: Malignant	Large intestine: Incidence
Badger *et al.*, 1940	Not stated	56	Not stated	6 Methyl-3:4-benz-phenanthrene	2–4 per cent solution in olive oil in food	Not stated	—	Carcinoma	1/10	—	—	—	—
Lorenz and Stewart, 1940	Strain A	>30	M. and F.	1, 2, 5, 6-dibenzan-thracene	Olive oil emulsion in drinking water (0·8 mg./day)	3–19 cm. from pylorus	—	Adeno-carcinoma	9/30	—	—	—	
	Strain A back cross	—	M.	20-Methylcholan-threne	Ditto	—	—	Ditto	4/10	—	—	—	—
Lorenz and Stewart, 1941	Strain C57 Brown, C57 Bl.	Not stated	Not stated	Ditto	Olive oil emulsion in drinking water	Not stated	—	,,	Not stated	Caecum. Upper part of colon	—	Adeno-carcinoma	A few
	C3H Dba	—	—	1, 2, 5, 6-dibenzan-thracene	Ditto								
White and Stewart, 1942	Strain C3H	>16	Not stated	20-Methylcholan-threne	Fatty solution incorporated in diet	8–25 cm. from pylorus	—	,, Haemangio-endothe-lioma	6*/50 11†/50.	— —	— —	— —	— —
	C	>29	,,	Ditto	Ditto	Ditto	—	Adeno-carcinoma Haemangio-endothe-lioma	4‡/32 21†/32	— —	— —	— —	— —
Foulds, 1947	Strain RIII	52	M.	2-Acetylamino-fluorene	In food	Not stated	—	Carcinoma	1/53	—	—	—	—
Miller and Pybus, 1956	Hybrid NBT × CBA	86	F.	20-Methylcholan-threne	Descendents of mice injected subcutaneously with 1 mg. in 0·1 c.c. sesame oil	—	—	—	—	Rec-tum Colon	— —	Tumour ,,	— —
		56	,,	Ditto	Ditto	Not stated	—	Tumour	—	—	—	—	—

* In addition, 18 mice had precancerous lesions of the small intestine. † Lesion present in mice of both sexes.
‡ In addition, 5 mice had precancerous lesions of the small intestine.

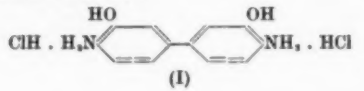
TABLE III.—*Methods of Administration and Source of Chemicals*

Chemical	Dose	Subcutaneous injection: Weekly dose per per mouse	Subcutaneous injection: Total dose per mouse at 52 wks.	Source of chemical	Number of mice at start	Whether vaccinated
Arachis oil	0·1 c.c.	0·2 c.c.	10 c.c.	Boots Ltd.	60	No
Benzidine	0·1 c.c. of 3 per cent suspension in arachis oil	6 mg.	312 mg.	British Drug Houses (AnalaR)	30 30	„ Yes
(I)—						
3 : 3′-Dihydroxybenzidine hydrochloride	Ditto	6 mg.	312 mg.	Synthesised in laboratory	30	No
1-Naphthylamine	Ditto	6 mg.	312 mg.	British Drug Houses (specially purified to be free of 2-naphthylamine)	30 30	„ Yes
2-Naphthylamine* B.D.H.	0·1 c.c. of 3 per cent suspension in arachis oil allowed to stand	6 mg.	312 mg.	British Drug Houses (specially prepared, (Bonser, 1943))	30	No
B.D.H.	Ditto, prepared fresh	6 mg.	312 mg.	Ditto	30	Yes
R.C.H.	Ditto	6 mg.	312 mg.	Prof. Bergel (specially purified)	30	„
(II)—						
0-Methyl-2-amino-1-naphthol hydrochloride	0·1 c.c. of 3 per cent solution in water	6 mg.	312 mg.	Synthesised in laboratory	30	„
(III)—						
Benzeneazo-2-anthrol	0·2 c.c. of 3 per cent suspension in arachis oil	12 mg.	624 mg.	Ditto	30	„
(IV)—						
Carmoisine	0·1 c.c. of 3 per cent suspension increased to 6 per cent after 6 months	6 mg. 12 mg.	468 mg.	Pointing, Hexham	30	No
(V)—						
Xylylazo-2-naphthol	0·1 c.c. of 3 per cent suspension in arachis oil	6 mg.	312 mg.	„	30	Yes

Chemical	Dose	Oral: Weekly dose per mouse	Oral: Total dose per mouse at 52 wks.	Source of chemical	Number of mice at start	Whether vacinated
(VI)—						
o-Tolylazo-2-naphthol	0·1 c.c. of 1·5 per cent suspension in arachis oil	3 mg.	156 mg.	Synthesised in laboratory	30	No
(VII)—						
Magenta	0·2 c.c. of 3 per cent suspension in arachis oil by stomach tube	12 mg.	624 mg.	British Drug Houses	60	Yes
(VIII)—						
Auramine	0·1 per cent in diet	14 mg.†	728 mg.	,, ,, ,,	30	,,
Xylylazo-2-naphthol	Ditto	14 mg.	728 mg.	Pointing, Hexham	30	,,
o-Tolylazo-2-napthol	Ditto	14 mg.	728 mg.	,, ,,	30	,,
(IX)—						
Rhodamine B	0·05 per cent in drinking water	17 mg.	884 mg.	,, ,,	30	,,
(X)—						
Sunset yellow	Ditto	17 mg.	884 mg.	,, ,,	30	,,
(XI)—						
Ponceau 2R	Ditto	17 mg.	884 mg.	,, ,,	30	,,

* For further details of preparation of solutions and chemicals see Bonser, Clayson, Jull and Pyrah (1956).
† It is assumed that each mouse eats approximately 2 g. of food per day and drinks 5 c.c. of water.

in arachis oil for 52 weeks. Thereafter the mice were allowed to survive as long as possible. For three reasons, this technique was varied: (*a*) to compare the effects of oral and subcutaneous administration in the case of certain chemicals which might be altered in the alimentary tract or metabolised in the liver; (*b*) to avoid inflammation of the subcutaneous tissues by irritant chemicals; and (*c*) to reduce labour. The methods of administration, dose, and source of the chemicals are given in Table III. The chemical formulae of the less well-known compounds are as follows :—



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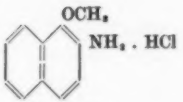

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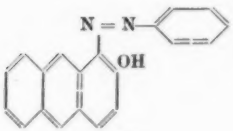

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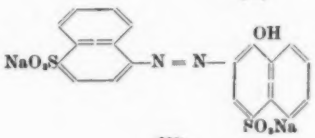

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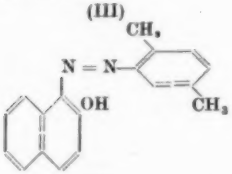

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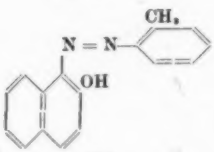

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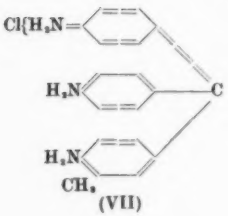

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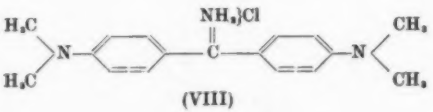

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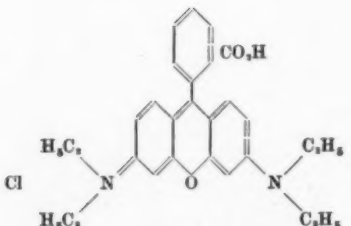

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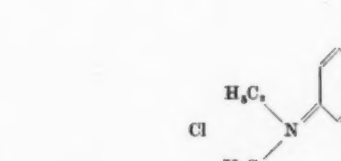

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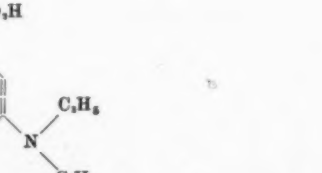

(XI)

Post-mortem *examination.*—At death a thorough examination of each mouse was made by searching for tumours in all organs. Microscopical examination of all suspicious lesions and of many apparently normal organs was undertaken.

EXPERIMENTAL RESULTS

The mortality at the beginning of the experiment, which took 3 years to complete, was very high. Improvement was obtained in some groups by vaccination against ectromelia.

Subcutaneous sarcomas.—With one exception, these occurred in mice receiving oily subcutaneous injections of the chemicals (Table IV). The 12 subcutaneous sarcomas induced with 2-naphthylamine have already been reported and discussed (Bonser, Clayson, Jull and Pyrah, 1956) as have the three induced by subcutaneous injection of *o*-tolylazo-2-naphthol (Bonser, Clayson and Jull, 1954). Of the three tumours induced in the subcutaneous tissues with benzeneazo-2-anthrol, two were spindle-cell sarcomas of vaso-formative type (Fig. 1), which resembled those described by Andervont (1950) as haemangio-endotheliomas following subcutaneous administration of crystalline *o*-aminoazotoluene, and one was a spindle-cell sarooma. They occurred at 34, 46 and 56 weeks in 2 males and 1 female. The three tumours induced with *o*-tolylazo-2-naphthol were spindle-cell sarcomas in females, at 46, 53 and 61 weeks respectively, as was the one tumour occurring at 56 weeks in the flank of a female mouse receiving oral auramine. The latter was not associated with parasitic infection.

Hepatomas.—A significant incidence of this type of tumour was obtained with benzidine, 2-naphthylamine, auramine and 0-methyl-2-amino-1-naphthol hydrochloride (Table IV). Single tumours were obtained with benzeneazo-2-anthrol and the injection of *o*-tolylazo-2-naphthol. Ten hepatomas occurred before and 16 after 70 weeks ; in the latter group were 6 induced by 2-naphthylamine. Those occurring after treatment with benzidine and 2-naphthylamine were large in size and sometimes multiple. Although no metastases were found, their structure frequently resembled that of malignant tumours. Those induced by auramine and 0-methyl-2-amino-1-naphthol were small and benign. It was rare for cholangiomatous areas to be present in addition to hepatomas.

Lymphomas.—This term is used to cover a'l the lesions arising in the reticular system. Using the classification of Dunn (1953), the two common tumours were lymphosarcoma and reticulum-cell sarcoma. The former varied greatly in its extent, and might be confined to the liver and spleen, or to the lymph glands, in an individual mouse. The latter arose usually in the abdominal cavity or in the liver and kidney. The tumours were composed of spindle cells and it was necessary to make a distinction between this type of tumour and the spindle-cell sarcomas arising locally in relation to subcutaneous injeotions. This was not difficult when the anatomical and microscopical appearances in each mouse were studied. This statement is in accord with the opinion of Dunn (1953).

Approximately one-third of untreated mice of this type used in this and other experiments suffer spontaneously from the disease, the females being more commonly affected than the males. Treatment by the chemicals under review did not significantly raise this incidence.

Intestinal tumours (Table V).—One year after the beginning of the experiment an intestinal carcinoma was found in the ileo-caecal region of a mouse injected

TABLE IV.—*Mortality and Incidence of Subcutaneous Sarcomas, Hepatomas and Leukaemia*

Chemical	Method of administration	Number of mice at start		Mice dying in weeks: 20–49 M.	20–49 F.	50–69 M.	50–69 F.	70–89 M.	70–89 F.	Over 90 M.	Over 90 F.	Total M.	Total F.	Lymphoma M.	Lymphoma F.
Arachis oil	Injected	60	D	1	1	1	4	6	2	1	2	9	9	1	4
Benzidine	,,	60	D	1	—	3	7	2	—	0	0	6	7	1	2
			H	1	—	1	3	2	—	—	—	4	3	—	—
3 : 3′-Dihydroxybenzidine	,,	30	D	4	—	3	3	1	3	0	0	8	6	0	4
1-Naphthylamine	,,	60	D	—	4	2	5	3	1	0	2	5	12	1	6
2-Naphthylamine	,,	90	D	13	7	6	6	1	5	1	2	21	20	3	8
			S	5	5	—	1	—	—	—	1	5	7	—	—
			H	—	—	1	—	1	2	1	2	3	4	—	—
Auramine	Oral	30	D	1	1	2	4	5	6	0	0	8	11	3	8
			S	—	—	—	1	—	—	—	—	—	1	—	—
			H	—	—	1	2	3	1	—	—	4	3	—	—
Magenta	,,	60	D	3	4	2	5	2	1	0	3	7	13	2	2
			H	—	—	—	—	—	—	—	1	—	1	—	—
0-Me-2-amino-1-napthol hydrochloride	Injected	30	D	1	3	3	2	4	5	0	0	8	10	5	6
			H	—	—	—	—	2	—	—	—	2*	—	—	—
Carmoisine	,,	30	D	—	3	1	3	—	5	0	0	1	11	0	7
Benzeneazo-2-anthrol	,,	30	D	4	1	4	7	2	4	0	0	10	12	0	4
			S	1	1	1	—	—	—	—	—	2†	1	—	—
			H	—	—	1	—	—	—	—	—	1	—	—	—
Rhodamine B	Oral	30	D	2	1	4	2	—	1	4	1	10	5	4	3
Xylylazo-2-naphthol	,,	30	D	4	4	3	4	4	2	0	0	11	10	5	8
	Injected	30	D	1	1	4	1	2	1	0	0	7	3	2	2
			H	—	—	—	—	1	—	—	—	1	—	—	—
o-Tolylazo-2-naphthol	Oral	30	D	3	1	2	5	7	2	0	2	12	10	1	5
	Injected	30	D	—	2	3	6	1	3	0	0	4	11	0	4
			S	—	1	—	2	—	—	—	—	—	3	—	—
Sunset yellow	Oral	30	D	2	—	3	6	4	4	0	0	9	10	3	6
Ponceau 2R	,,	30	D	2	1	5	2	1	2	0	0	8	5	6	1

D = deaths. S = subcutaneous sarcomas. H = hepatomas.
* One liver with advanced cholangioma also.
† One squamous carcinoma of orbit in addition.

TABLE V.—*Mortality of Mice in which the Ileo-caecal Region and the Pylorus were Examined Microscopically and the Incidence of Intestinal Tumours and Pyloric Polyposis*

Chemical	Method of administration	Survivors dying at weeks: 20–49 M.	20–49 F.	50–69 M.	50–69 F.	70–89 M.	70–89 F.	Over 90 M.	Over 90 F.	Total intestinal tumours: Benign	Malignant	Total survivors M.	Total survivors F.	Mice with polyposis of stomach M.	Mice with polyposis of stomach F.
Arachis oil	Injection	—	—	—	0/3	1B/4	0/2	0/2	0/2	1	0	6	7	2	1
Benzidine	,,	—	—	0/1	0/6	0/2	—	—	—	0	0	3	6	0	0
3 : 3′-Dihydroxybenzidine	,,	—	—	1B/1	0/1	—	0/3	—	—	1	0	1	4	0	0
1-Naphthylamine	,,	—	—	0/2	0/2	0/3	—	0	1B/2	1	0	5	4	2	0
2-Naphthylamine	,,	—	—	0/5	0/3	—	1C/3	0/2	1B/2	1	1	7	8	3	3
Auramine	Oral	0	0/1	1C/2	0/2	0/3	0/3	0	0	0	1	5	6	1	1
Magenta	,,	—	—	0/1	0/2	0/2	0/1	0	0/2	0	0	3	5	1	1
0-Methyl-2-amino-1-naphthol hydrochloride	Injection	0/1	0/3	1B/1	1B/2	4B/4	2B/3	—	—	8	0	6	8	0	0
Carmoisine	,,	—	—	1B/1	0/1	—	0/4	—	—	1	0	1	5	0	2
Benzeneazo-2-anthrol	,,	—	—	0/3	0/5	0/2	0/2	—	—	0	0	5	7	0	0*
Rhodamine B	Oral	—	—	1B, 1C/3	0/1	—	—	0/4	—	1	1	7	1	2	1
Xylylazo-2-naphthol	,,	1B/4	1B/2	2B/3	0/4	0/2	—	—	—	4	0	9	6	5	0
	Injection	0/1	—	0/3	—	0/1	—	—	—	0	0	5	0	2	1
o-Tolylazo-2-naphthol	Oral	1B, 1C/3	0/1	1B/1	0/3	7B/6	0/3	0	0/2	9	1	10	9	4	1
	Injection	—	—	1C/1	1B, 1C/2	0/1	0/3	—	—	1	2	2	5	0	0
Sunset yellow	Oral	—	—	0/2	1B/5	—	—	—	—	1	0	2	5	1	1
Ponceau 2R	,,	—	—	1B/2	1B/1	—	—	1B/1	1B/2	4	0	3	3	2	1

* One squamous papilloma and one squamous carcinoma of the stomach.
Numerators represent number of mice with benign (B) or malignant (C) intestinal tumours.

with *o*-tolylazo-2-naphthol. Thereafter, the intestinal tract was searched more thoroughly for possible tumours and all suspicious areas were examined microscopically. In addition to cutting sections of the ileo-caecal region (Table V), 259 pieces of small intestine and 93 of large intestine were cut and examined.

It was decided to accept as without demonstrable action on the intestinal tract those compounds where one intestinal tumour or no tumours were present in the group. These are: arachis oil, benzidine, dihydroxybenzidine, 1-naphthylamine, auramine, magenta, carmoisine, benzeneazo-2-anthrol and sunset yellow. With these chemicals the incidence of polyps (only one of which, in an auramine mouse, was malignant) was 4 out of 31 males and 2 out of 49 females (Table V). The remaining compounds, i.e. 2-naphthylamine, 0-methyl-2-amino-1-naphthol hydrochloride, rhodamine B, xylylazo-2-naphthol, tolylazo-2-naphthol and ponceau 2R, each produced more than one intestinal tumour in the group. In total, there were 23 intestinal tumours in 49 males and 10 in 40 females (Table VI). Of these, five tumours were intestinal carcinomas.

Morbid anatomy and histology.—The site of election was the ileo-caecal junction (35 out of 39 tumours). Where the precise origin was detectable, it was usually from the wall of the caecum, but some tumours seemed to start exactly at the junction and others were multifocal, arising both at the junction and elsewhere. One benign polyp arose in the ileum at a little distance from the junction, and another in the colon near to the junction with the caecum. The site of two carcinomas was in the colon about 1½ inches from the anus. The tumours could be detected either by observation of a thickening of the region or by the presence of small shining cysts on the serosal surface. Some of the small tumours were not detected with the naked eye.

Structure of the polyps.—These were usually sessile adenomas, raised from the mucous surface and composed of regular intestinal glands, lined by high mucus-secreting epithelium, in which the nuclei are markedly hyperchromatic (Fig. 2 and 3). Dipping of the glands through the muscularis mucosae and the muscular wall of the intestine into the mesentery was a frequent feature causing the cysts seen externally with the naked eye (Fig. 4). Other cysts were formed by oblique cutting of pockets of the intestinal lumen caused by the projecting polyp and these sometimes contained faeces or portions of tapeworm. Sometimes there was a granulomatous reaction in the mesentery, thought, to be due to the bursting of one or more of the cysts. Similar tumours were described by Miller and Pybus (1956).

Structure of the carcinomas.—All but one of the 6 tumours of this type could be seen to arise in pre-existing polyps. In 2, there was no muscle invasion (Fig. 5); in 2 there was complete penetration of the muscular coat by mucus-forming irrregular cystic glandular spaces; in 2 there was anaplastic invasive growth penetrating into the mesocaecum and associated with serosal lymphatic permeation and metastasis to regional lymph nodes (Fig. 6). Adjacent to both these tumours were benign cysts of the type described above as forming the deep part of benign papillomas of the caecal region. The finding of these cysts was regarded as evidence of the previous presence of a benign tumour.

Pyloric polyposis.—This is a proliferative condition associated with overgrowth of pyloric glands, which penetrate the muscularis mucosae and the muscular coat, but do not invade lymphatic vessels nor metastasise. This condition is spontaneous in the I and other strains (Stewart and Andervont, 1938) and has

TABLE VI.—*Analysis of Benign and Malignant Tumours of the Intestine*

Compounds which produced more than one intestinal tumour				
	Intestinal tumours in:			
Chemical	Males	Females	Benign polyps	Carcinomas
2-Naphthylamine	0/7	2/8	1	1
0-Methyl-2-amino-1-naphthol hydrochloride	5/6	3/8	8	0
Rhodamine B	2/7	0/1	1	1
Xylylazo-2-naphthol—				
Injected	0/5	—	4	0
Oral	3/9	1/6		
Tolylazo-2-napthol—				
Injected	1/2	2/5	10	3
Oral	10/10	0/9		
Ponceau 2R	2/3	2/3	4	0
Total	23/49	10/40	28	5

Compounds which produced one intestinal tumour or less				
	Intestinal tumours in:			
Chemical	Males	Females	Benign polyps	Carcinomas
Arachis oil	1/6	0/7	1	0
Benzidine	0/3	0/6	0	0
3 : 3′-Dihydroxybenzidine	1/1	0/4	1	0
1-Naphthylamine	0/5	1/4	1	0
Auramine	1/5	0/6	0	1
Magenta	0/3	0/5	0	0
Carmoisine	1/1	0/5	1	0
Benzeneazo-2-anthrol	0/5	0/7	0	0
Sunset yellow	0/2	1/5	1	0
Total	4/31	2/49	5	1

recently been described by Miller and Pybus (1956) in older mice of crosses between the NBT and CBA strains. The lesion occurred in control and treated mice of the present experiment, and was more common in males than in females. It is usually non-malignant, though Miller and Pybus described one tumour which they thought was an adenocarcinoma.

Other tumours.—A squamous carcinoma of the orbit occurred at 63 weeks in a male mouse injected with an oily suspension of benzeneazo-2-anthrol. A squamous papilloma of the lower end of the oesophagus and a squamous carcinoma of the cardiac end of the forestomach also occurred in 2 females at 58 and 56 weeks respectively in the group treated wth this chemical. Occasional mammary carcinomas and granulosa-cell ovarian tumours occurred in control and other groups. One spindle-cell sarcoma of the uterus occurred at 70 weeks in the xylylazo-2-naphthol group and one osteogenic sarcoma of the jaw at 74 weeks in a female treated with sunset yellow.

DISCUSSION

Single early benign intestinal polyps were found in groups of mice treated with arachis oil, 3 : 3′-dihydroxybenzidine, 1-naphthylamine (free from 2-naphthylamine), and carmoisine. One small hepatoma was seen at 101 weeks in a female treated with magenta and an osteogenic sarcoma of the jaw occurred in a mouse treated with sunset yellow. As all these tumours might well be casual occurrences these six compounds are regarded as being without carcinogenic activity in this experiment. Case and Pearson (1954) stated that the manufacture of magenta had an " occupational hazard of causing tumour of the urinary bladder attached to it." No carcinogenic properties have been demonstrated in magenta in the present experiment, where a high dose was used but only a few mice survived to a late period.

Baker (1950) claimed that 3 : 3′-dihydroxybenzidine, recovered from the urine of industrial workers, was carcinogenic to the mouse when given subcutaneously in oily solution, producing benign and malignant tumours in the bladder and the liver. As the author stated that the chemical was not completely purified, as benign and possibly malignant hepatomas were also observed in untreated control animals and as the illustrations of the tumours are quite inconclusive, the value of the observations is doubtful.

Case, Hosker, McDonald and Pearson (1954) brought evidence to show that 1-naphthylamine was a cause of bladder tumours in industry. They thought it possible that the 2-naphthylamine content, always present in crude samples, was not the sole causative agent of the industrial tumours. The present experiment brings no further evidence that the purified compound is carcinogenic either to the bladder or to any other organ.

The following compounds were regarded as showing carcinogenic activity : benzidine, 2-naphthylamine, auramine, *o*-tolylazo-2-naphthol and benzeneazo 2-anthrol.

Benzidine was shown by Spitz, Maguigan and Dobriner (1950) to induce hepatomas; intestinal carcinomas and acoustic duct carcinomas in the rat. Baker (1953) was also able to induce these types of tumour in Slonaker rats, and he claimed 2 squamous carcinomas of the forestomach and one papilloma and 2 squamous carcinomas of the urinary bladder. Tumour induction in the dog requires a long latent period and yields only a low incidence of tumours. Spitz

(personal communication) found that the 3 longest survivors of 7 dogs given large doses of benzidine by mouth developed papillomas and carcinomas of the bladder after 7, 8 and 10 years respectively. Of 4 dogs treated similarly in Leeds, two survived for 8½ and 9 years and had no bladder tumours at death (unpublished observation). An invasive bladder carcinoma was induced after 2¼ years in one rabbit in Leeds (unpublished observation), among 7 treated by oral administration of benzidine. Baker (1950) described 5 hepatomas—4 benign and 1 malignant —in 9 mice of Oldham stock which had received subcutaneous injections of an oily suspension of 300 mg. of benzidine base 3 times per week for 45 weeks. Five of 17 untreated mice developed 3 benign and 2 doubtfully malignant hepatomas in the same time period. The dose reported by Baker was found in the present experiment to be greatly in excess of the maximum which could be tolerated by our mice. Our tumours were confined to the liver. They occurred in both sexes between 50 and 79 weeks of treatement.

The tumours induced with 2-naphthylamine have already been discussed (Bonser *et al.*, 1956) and reasons given for regarding the subcutaneous sarcomas as due to the development of carcinogenic properties in oily solutions on standing. Hepatomas occurred even when the water-soluble hydrochlorides were injected and this was regarded as being due to the action of 2-naphthylamine or its metabolites on the liver. All but one occurred after 70 or more weeks of treatment, i.e. late in the experiment. In addition, one benign polyp and one carcinoma arose at the ileo-caecal junction.

The claim for carcinogenic properties in auramine lies in the 7 hepatomas (4 in males and 3 in females) occurring in 19 mice. The one carcinoma of the colon in a male mouse treated for 67 weeks is regarded as probably fortuitous and the origin of the subcutaneous sarcoma following oral administration is unexplained, though it might have been caused by the chemical.

o-Tolylazo-2-naphthol was administered by injection and feeding. One small intestinal polyp and 2 carcinomas occurred after injection (Bonser, Clayson and Jull, 1954) and 9 polyps and one carcinoma after feeding. In the latter group, only the males were affected and all the polyps but one were at the ileo-caecal junction and were of the characteristic cystic type. In the benzeneazo-2-anthrol group, were 3 subcutaneous sarcomas, 2 of vaso-formative type, usually classified by other authors as haemangio-endotheliomas (Foulds, 1930 ; White and Stewart, 1942 ; Andervont, 1950). White and Stewart suggested that their tumours occurred along the line of drainage of oral methylcholanthrene from the intestinal tract. The present tumours could either be regarded as occurring at the site of injection of the chemical or in tissues likely to be receiving lymph from the injected area. These were the only tumors of this type in the whole experiment and therefore they are regarded as being specifically related to this chemical. One squamous papilloma and one squamous carcinoma of the forestomach, and one squamous carcinoma of the orbit were also observed in the group treated with benzeneazo-2-anthrol. The significance of the last-named tumour is doubtful.

0-Methyl/-2-amino-1-naphthol hydrochloride, xzylylazo-2-naphthol, ponceau 2R and rhodamine B are chemicals of which the carcinogenic activity is in doubt. When the mice were treated with 0-methyl-2-amino-1-naphthol hydrochloride by subcutaneous injection, 2 hepatomas were found in male mice but the outstanding feature was the 8 benign polyps, all situated at the ileo-caecal junction and all diagnosed on naked-eye examination of the exterior by the presence of subserous

and intra-muscular cysts. These occurred between 55 and 78 weeks, i.e. well within the age when carcinomas had developed in other groups. Therefore, although the high incidence of benign tumours suggests that the compound is not without tumour-inducing properties, the absence of malignant change leaves its carcinogenic nature in doubt. Xylylazo-2-naphthol was administered by injection and feeding. No intestinal tumours occurred in 5 injected survivors but there was one hepatoma ; 4 benign intestinal polyps were induced when the dye was fed. These were found at the ileo-caecal junction and 3 of them showed the characteristic cysts. Four benign caecal polyps were induced in males and females with ponceau 2R.

In regard to the intestinal tumours as a whole, there was a marked predilection for the male sex (27 out of 80 males and 12 out of 89 females). There was histological evidence that carcinomas arose in polyps, but none of the polyps in mice treated with 0-methyl-2-amino-1-naphthol hydrochloride, xylylazo-2-naphthol or ponceau 2R became malignant within the period of the experiment. The preponderance of tumours at the ileo-caecal junction suggests that stasis of the intestinal contents is a factor concerned in carcinogenesis.

SUMMARY

An experiment is described in which stock albino mice, bought from a dealer, were used to test the carcinogenic properties of 14 chemicals and the vehicle of administration.

The chemicals were either : (1) dyes (auramine and magenta), the manufacture of which constitutes an industrial hazard ; (2) dyes (carmoisine, rhodamine B, sunset yellow, ponceau 2R, xylylazo-2-naphthol and tolylazo-2-naphthol) formerly or at present in use as food colorants ; (3) dyestuffs intermediates (benzidine, 1-naphthylamine and 2-naphthylamine) ; or (4) ortho hydroxyamines (3 : 3′-dihydroxybenzidine and 0-methyl-2-amino-1-naphthol hydrochloride) related to known metabolites of aromatic amines.

The chemicals were administered either by subcutaneous injection of oily solutions or suspensions, or orally in the diet, in the drinking water or by stomach tube.

The carcinogenic activity was assessed by the occurrence of subcutaneous sarcomas, hepatomas, and intestinal polyps and carcinomas. Lymphomas and pyloric polyposis occurred spontaneously and their incidence was not enhanced by the treatment.

Arachis oil, 3 : 3′-dihydroxybenzidine, 1-naphthylamine (free from 2-napthylamine) and carmoisine induced no tumours. The one hepatoma obtained with magenta, and the one osteogenic sarcoma of the jaw with sunset yellow were regarded as fortuitous occurrences, not related to the treatment.

Benzidine, 2-naphthylamine and auramine were regarded as carcinogenic on the grounds of the occurrence of hepatomas. Two caecal polyps, one benign and one malignant, in mice treated with 2-naphthylamine and one carcinoma of the colon in an auramine-treated mouse were regarded as probably induced by the chemical. The cause of the one subcutaneous sarcoma after oral administration of auramine is unexplained. *o*-Tolylazo-2-naphthol caused 13 intestinal tumours, 10 benign and 3 malignant, in 26 mice. The degree of malignancy of the tumours in this group was greater than with any other chemical, lymph-node metastases

being present in 2 cases. There was a marked predilection for the male sex. Intestinal tumours followed both feeding and subcutaneous injection, the latter method being the cause of 3 subcutaneous sarcomas. Benzeneazo-2-anthrol injected subcutaneously in oil caused 3 subcutaneous sarcomas, 2 of which were of unusual vasoformative type, one squamous papilloma and one squamous carcinoma of the forestomach, and a squamous carcinoma of the orbit, the latter probably fortuitous.

The carcinogenicity of the remaining chemicals, i.e. xylylazo-2-naphthol, 0-methyl-2-amino-1-naphthol, ponceau 2R and rhodamine B remains in doubt, the first three because none of the intestinal polyps had progressed further than the benign stage and the last because, although one tumour was malignant, only 2 tumours were found in 8 mice.

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THE PRODUCTION OF MALIGNANT TUMOURS BY COBALT IN THE RAT

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A DESCRIPTION of the effect of cobalt on chick cells in tissue culture and a preliminary report on the production of malignant tumours by cobalt in the rat have already been published (Heath, 1954*a*, 1954*b*). Since then the remainder of the animals in the first series described in that report have either developed tumours or have died; a second series of rats has been similarly treated with cobalt and the animals kept either until tumours developed or until death. Animals in which tumours developed were killed when the tumours had reached a size beyond which suffering was likely to occur. This present paper gives the experimental details together with the pathological reports on the tumours and some cytological observations. The author is indebted to his colleague Dr. A. Glücksmann for the pathological examinations and reports.

MATERIALS AND METHODS

Two series of rats are described here:

Series I injected with cobalt on July 17, 1953 and all killed for diagnosis or dead by November 9, 1955 (Table I).

Series II injected with cobalt on February 16, 1954 and all killed for diagnosis or dead by February 22, 1956 (Table II).

In both series rats of the hooded strain, aged 2–3 months, were used for injection. Series I comprised 10 males and 10 females, and series II, 10 females. 0·028 g. of spectroscopically pure cobalt metal powder shaken into suspension in 0·4 ml. of fowl serum was injected into the thigh muscle of each animal of both series from the medial aspect of the leg, the left leg being used for series I and the right leg for series II. The cobalt powder was described as 400 mesh by the suppliers and on microscopic examination was found to consist of mainly rectangular particles of a somewhat laminated or fibrous appearance. The particle size ranged from 3·5 μ × 3·5 μ to 17 μ × 12 μ with large numbers of long narrow particles of the order of 10 μ × 4 μ. Clumps of particles measuring up to 100 μ × 100 μ were also present. In series I, 10 control rats were injected at the same site with 0·4 ml. fowl serum alone. In series II, 5 control rats were injected in the same site with 0·028 g. zinc powder in 0·4 ml. fowl serum and another 5 rats with 0·028 g. tungsten powder in 0·4 ml. fowl serum. The zinc powder consisted of spherical particles ranging from 1·5 μ diameter to 44 μ diameter with most of the particles having diameters between 4 μ and 20 μ. The zinc particles showed no tendency to form clumps. The tungsten particles were smooth and approximately rectangular in shape with sizes ranging from 5 μ × 5 μ to 50 μ × 50 μ with most

of the particles lying between 8 $\mu \times 12$ μ and 10 $\mu \times 30$ μ. The tungsten particles were occasionally grouped in clumps measuring up to 170 $\mu \times 170$ μ.

RESULTS

In each series there appeared to be little or no immediate local reaction to the injections and no systemic effect, and within a few days the injection site could not be detected either by inspection or palpation. In series I and II the first tumours were apparent at 5 months and the last ones by 12 months. All the tumours occurred at the injection site, a high proportion of them being rhabdomyofibrosarcomata and others sarcomata of various types, e.g. round cell, fibro- and polymorphous sarcomata as described in Tables I and II. In these latter sarcomata it cannot be stated with certainty that a rhabdomyosarcomatous component was entirely absent as the tumours were so large that a complete microscopic examination was precluded. Microphotographs of typical tumour sections are shown in Fig. 1–3. One of the original tumours in series I (rat no. 5693) was used for transplantation into other rats of the same strain and at the same site ; this tumour is now in its 27th passage and transplants grow to optimum size in 4–5 weeks. Even when measuring approximately $4 \times 4 \times 4$ cm. the transplanted tumours remain firm and white with only a little central necrosis. No tumours occurred in any of the controls except for one malignant lymphoma in a zinc-injected rat. This disseminated tumour did not appear at the injection site but probably originated in the thymus, and is of a type which occasionally arises spontaneously in rats of this strain after a variety of treatments. It cannot therefore be regarded as a specific result of the zinc treatment.

It is seen that the tumours induced by the cobalt arose from the connective tissue present in the thigh muscle or from the striated muscle itself or from both. The sarcomata of these series are similar to those induced by many other carcinogenic agents. Malignant changes in muscle however are rare in experimental tumour induction and it is interesting that cobalt should have induced typical rhabdomyosarcomata in the rats. The malignant cells which appear to be derived from the striated muscle are elongated and vary considerably in width and still more in length (Fig. 2). They may be multinucleated (Fig. 3) and mitoses are common. Many of the mitoses show abnormalities such as multipolarity, aberrant chromosomes, failure of the chromosomes to separate in anaphase, and polyploidy. The nuclei of the interphase cells vary greatly in size and shape ; many are hyperchromatic and have abnormally large nucleoli. The presence of fine parallel longitudinal fibrils in the cytoplasm (Fig. 3) is interpreted as an indication that these cells are rhabdomyosarcoma cells. They can readily be distinguished from those of striated muscle regenerating after simple trauma, for in these cells the nuclei are rather uniform in size, hyperchromatic and giant nuclei are absent, mitoses are rare and when observed appear normal. Such non-malignant regenerative changes in skeletal muscle have been described in the older literature and more recently by Le Gros Clark (1946).

Close examination of sections of the cobalt-induced tumours fixed in Carnoy's fluid and stained with methyl green-pyronin reveals some mitotic cells with persistent nucleoli up to and including late metaphase (Fig. 4), but unlike the experimental findings in the cobalt-treated cultures of heart tissue from 10–12-day-old chick embryos (Heath, 1954*a*) no persistent nucleoli have been found in

TABLE I.—*Series I : Rats Injected July* 17, 1953

Rat No.	Time to appearance of tumour in weeks	Time to autopsy in weeks D = died K = killed	Size of tumour at autopsy in cm.	Pathological report
Male				
5682	—	21 (D)	0	
5683	21	26 (K)	3×3×2	A highly cellular and fairly undifferentiated rhabdomyofibrosarcoma with a lymph node metastasis.
5684	21	26 „	3×2×2	A cellular rhabdomyofibrosarcoma with slightly better differentiation.
5685	33	44 „	4×3×3	A very cellular round-cell sarcoma with formation of multinucleate symplasms suggesting a myoblastic origin.
5686	41	44 „	4×3×3	A fairly differentiated rhabdomyofibrosarcoma.
5687	—	99 (D)	0	
5688	—	119 (K)	0	
5689	—	119 „	0	
5690	—	107 „	0	
5691	—	107 „	0	
Female				
5692	—	23 (D)	0	
5693	27	32 (K)	4×3×3	The tumour is a moderately differentiated fibrosarcoma with giant cell formation, fairly dense fibres and some myomatous regions. There is no definite evidence of the tumour having arisen in muscle tissue.
5694	32	35 „	4×3×3	A cellular fibrosarcoma.
5695	34	39 „	3×3·5×3	A fairly cellular fibrosarcoma with numerous mono- and multinuclear giant cells.
5696	34	51 „	4·5×4×4	A highly undifferentiated, predominantly cellular rhabdomyosarcoma.
5697	51	58 „	3×2×2	A moderately differentiated rhabdomyofibrosarcoma.
5698	—	58 (D)	0	
5699	—	122 (K)	0	
5700	—	73 (D)	0	
5701	—	121 „	0	

TABLE II.—*Series II : Rats Injected February* 16, 1954

Rat No. Female	Time to appearance of tumour in weeks	Time to autopsy in weeks D = died K = killed	Size of tumour at autopsy in cm.	Pathological report
5718	20	24 (K)	4×3·5×2·5	A moderately differentiated rhabdomyofibrosarcoma.
5719	20	28 „	4·5×3·5×3·5	A fairly cellular rhabdomyofibrosarcoma.
5720	39	44 „	5×4·5×4	A moderately differentiated rhabdomyofibrosarcoma.
5721	—	51 „	3×3×3	A fairly undifferentiated rhabdomyofibrosarcoma.
5722	—	105 „	—	An abscess in the region of the injection site.
5723	10	10 „	3×3×2	A haemorrhage showing attempts at organisation of the clot.
5724	—	28 „	4×4×3·5	A highly cellular sarcoma of probably myoblastic origin containing a rhabdomyosarcomatous component.
5725	32	35 „	5×4×4	A cellular fibrosarcoma.
5726	—	51 „	4×4×4·5	A fairly undifferentiated rhabdomyofibrosarcoma.
5727	—	57 „	4×4×3·5	A polymorphous cell sarcoma with numerous giant cells probably arising in striated muscle.

the tumour cells in stages of mitosis later than metaphase. This could be due in part to the improbability that any given section through a cell in anaphase or telophase would include both the chromosomes and the nucleoli; in a tissue culture all the contents of a particular cell can been seen at once and not, as in sections, only those structures which lie in a given plane.

Giant cells are present in the tumours (Fig. 3, 5) but they are not of the same type as those observed in the cobalt-treated tissue cultures of chick heart and described previously (Heath, 1954*b*). The tumour giant cells are of the type often encountered in sarcomata and display a large irregular nucleus or a collection of nuclei.

It is not yet known whether the cytological effects produced *in vitro* by the cobalt are forerunners of the cobalt-induced malignant change *in vivo*. Further experiments are now in progress to try to elucidate this point.

DISCUSSION

Various metals are known to be capable of inducing malignant tumours, including nickel (Hueper, 1952), beryllium (Barnes, 1950), arsenic (Pye-Smith, 1913; Currie, 1947), chromium (Hueper, 1942), and now cobalt. The experiments described in this report were undertaken because the changed cells produced by cobalt treatment of tissue cultures suggested a possible malignant alteration. The results obtained show that cobalt metal is capable of producing malignant tumours in the muscles of a high proportion of the rats treated, owing to neoplastic changes both in connective tissue and striated muscle. When it became evident that cobalt was a strong carcinogen, a search of the literature revealed that Schinz and Uehlinger (1942) had reported malignant tumours in two out of a number of rabbits injected intrafemorally with cobalt metal, although at the time this was unknown to the present author. One of these tumours was a spindle cell sarcoma at the injection site arising six years after injection, and the other, at a site remote from the injection, was a multiple adenocarcinoma of the lung with peritoneal metastases.

The results described in this paper must inevitably raise again the question of what is the causative agent of the high incidence of pulmonary cancer in the Schneeberg miners (Currie, 1947). The radioactive, as well as the arsenic content of the Schneeberg ores have both been incriminated as the carcinogenic factors. Since a typical analysis of the Schneeberg mine dust shows 0·19 per cent of cobalt

EXPLANATION OF PLATES

All figures are photomicrographs of sections of cobalt-induced rat tumours. The sections are stained with haematoxylin and eosin after Zenker fixation, except the section shown in Fig. 4 which is stained with methyl green and pyronin after Carnoy fixation. Magnification of Fig. 1 and 2 is × 450, and of Fig. 3–5, × 950.

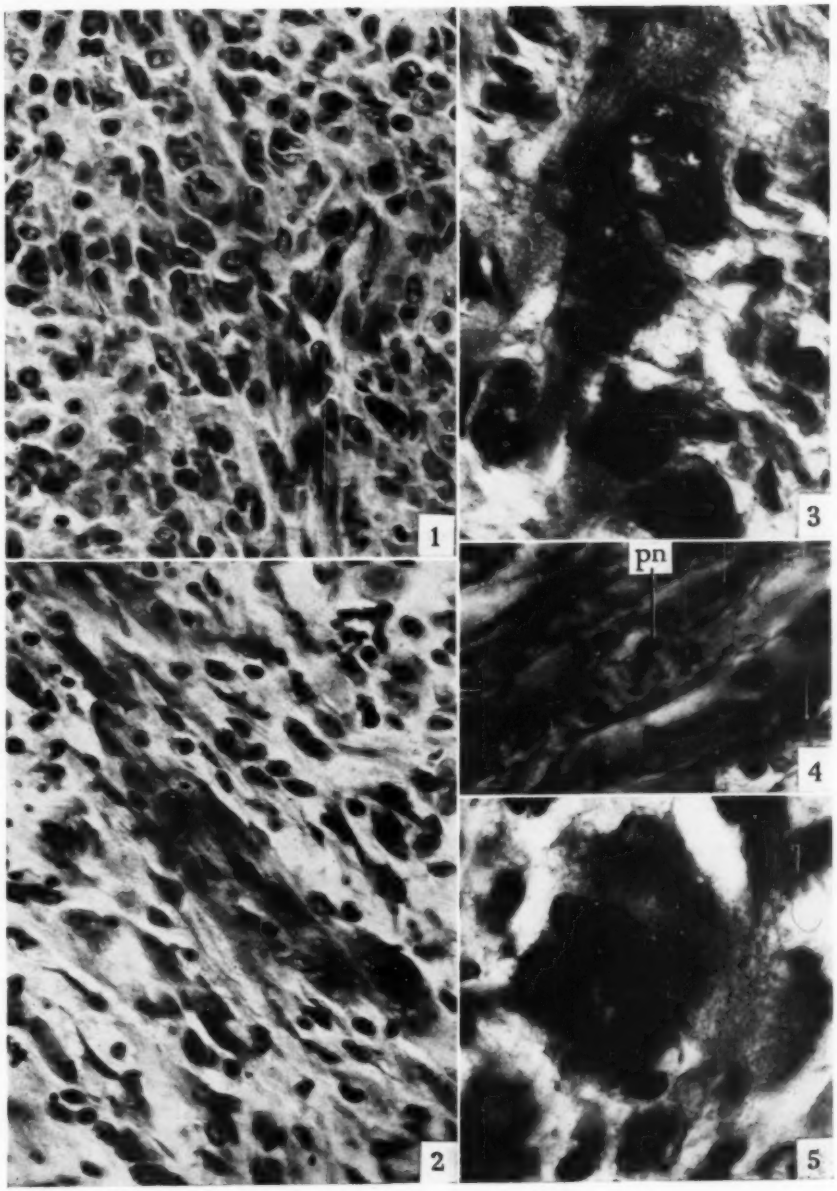
FIG. 1.—Rat 5683. Fast growing edge of tumour showing numerous mitotic figures.

FIG. 2.—Rat 5684. Elongated cells of myoblastic type; one is in mitosis.

FIG. 3.—Rat 5684. Multinucleate cell of the myoblastic type with fine longitudinal striations in the cytoplasm suggesting myofibrils.

FIG. 4.—Rat 5694. Tumour cell in metaphase with a persistent nucleolus (p.n.) shown as a dark spherical body attached to the metaphase plate. In the section this spherical body is stained red and the chromosomes green.

FIG. 5.—Rat 5693. A multinucleated giant cell in the tumour area.



Heath.

arsenide and 0·08 per cent of nickel-cobalt (Currie, 1947) it now seems likely that the cobalt might also be held suspect as believed long ago by Osler and others (Currie, 1947). It also seems desirable to re-investigate the possible carcinogenic hazards of inhalation of cobalt-bearing dusts by workers in industry. Some work on these lines with negative results in relation to the cemented tungsten carbide industry where cobalt is used as a cement has already been reported (Miller, Davis, Goldman and Wyatt, 1953; Lundgren and Swennson, 1953; Fairhall, Keenan and Brinton, 1949).

SUMMARY

The production of malignant tumours in 17 out of 30 rats injected intramuscularly with pure cobalt metal powder is described. The tumours all occurred at the injection site and a high proportion of them were rhabdomyofibrosarcomata, the remainder being sarcomata of various types.

The relationship of these findings to previously described effects of cobalt on chick cells in tissue culture is discussed.

The author is grateful to his colleagues Dr. Honor B. Fell, Dr. A. Glücksmann, and Dr. W. Jacobson for many helpful discussions on this work, and to his technician, Miss Valerie Galer, for her skilful and painstaking assistance in these experiments. The work was supported by the British Empire Cancer Campaign.

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THE EFFECTS OF FRACTIONATED X-IRRADIATION ON THE GROWTH OF SARCOMA 37 IN MICE

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IT has been shown by Warren and Whipple (1923) that small aliquot doses of X-irradiation administered over periods of up to 5 days, produced the same degree of destruction of the intestinal mucosa of dogs as a single total dose. This summation effect was lost if the intervals were spread over a longer period. In the treatment of breast cancer Cohen (1952) has suggested that it is not possible to improve the therapeutic ratio by prolonged daily fractionations. Quimby and MacComb (1937) have shown that if the total dose and total treatment time are constant, the number of fractions is not important. Du Sault (1956) in a review of the effects of fractionated doses in radiation therapy, has postulated a latent period within which any type of fractionation will produce the same effect as a single total dose. The effect of fractionated doses administered after this period—during the period of recovery—depends on the degree of recovery from the preceding dose.

Lamarque and Gary-Bobo (1955) have assessed the effects of variations in the time interval between two equal fractions, on the survival of eggs of *Bombyx mori*. In this work it was shown that a maximum effect was produced by a single total dose, and this effect decreased progressively when the dose was fractionated with time intervals up to 24 hours; an increase was then shown with time intervals of 2 and 4 days. Heeren (1948) has shown that the reticulocyte count in mice is lowered to a greater degree by continuous than by discontinuous irradiation. In studies on erythema Reisner (1932) showed that the effect of a single dose was greater than that of fractionated doses. Schöttelndreyer (1949), also studying erythema, showed that the biological effect of a single irradiation of 631 r was equivalent to 2064 r given in 12 daily fractions. Fogg and Cowing (1953) however, report that fractionated doses of 294 r produce a greater effect on spermatogonia and spermatocytes of mice than a single total dose.

The present study was undertaken to examine the effects of fractionated X-irradiation at varying time intervals on the growth of a transplantable tumour in mice.

MATERIALS AND METHOD

The transplantable tumour used in this study was Sarcoma 37, inoculated into RIII strain mice. This tumour was selected for the regularity of its growth rate, and absence of spontaneous regressions when inoculated into this strain. The tumours for each experiment were obtained by inoculating 1 mm.^{3} portions, from the cortex of an actively growing parent tumour, subcutaneously into the right flank of female mice. Each experiment consisted of control and treated

groups, each group consisting of at least ten mice. The growth rates of the control tumour groups were closely comparable. The tumours were measured at two-day intervals with calipers, the tumour area being taken as the product of the major and minor axes. This product is adequate for the relative comparison of tumour growth, although the sizes compared are not absolute.

Treatment began on the fifth day after inoculation, when the tumours were about 18 mm.^{2} in area. At each irradiation treatment half the dose was administered from each of two opposing directions, the centre of the tumour being situated 23·5 cm. from the anode and 1·5 cm. from the edge of the applicator. The mice were strapped down lying on their left sides so that the tumours were situated vertically; lead body shields protected them from scattered irradiation. X-rays were produced by a Siemens Stabilivolt Constant Potential Unit at 116 kV and 8 mA. No filters were used. The tumour dose rate was 130 r/min.

1000 r, a dose having a temporary inhibitory effect on the growth of Sarcoma 37, was chosen as the total dose. This dose was fractionated into 4 doses of 250 r in each experiment, and the time intervals used between doses were 15 minutes, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours respectively. The sensitivity of this tumour, under these conditions, to single doses of X-irradiation lies in the range of a detectable growth inhibitory effect at 150 r, to a tumour LD_{50} effect at 3500 r.

The anti-mitotic effects of 1000 r and 250 r were estimated by mitotic counts on the cortical region of the tumour.

RESULTS

Comparable growth rates were obtained for tumours subjected to fractionated doses at time intervals of 12 hours or less (Fig. 1). These growth rates approached

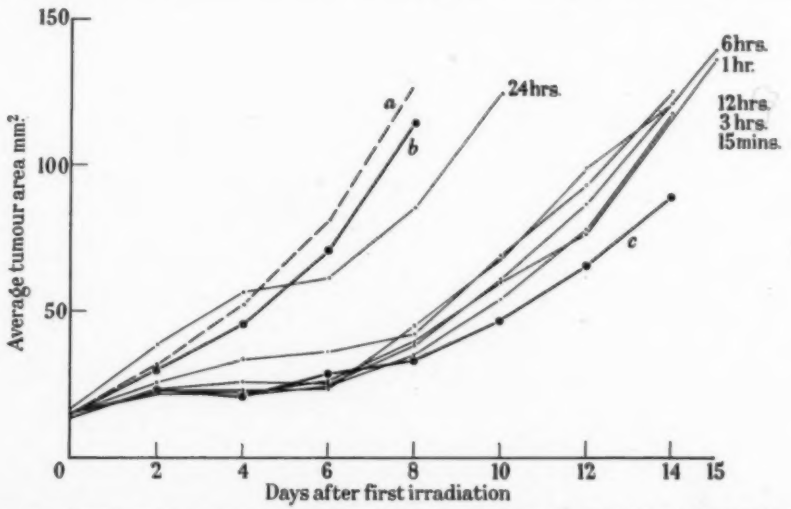


FIG. 1.—Growth rates of Sarcoma 37 after single doses of 250 r (*b*) and 1000 r (*c*), and after four divided doses of 250 r at intervals of ¼, 1, 3, 6, 12, 24 hours. Growth rate of control tumours is represented by the broken line (*a*).

the effect produced by the single total dose of 1000 r, but did not exceed this degree of inhibition. The tumours irradiated at 24-hour intervals showed a degree of inhibition slightly in excess of the value for a single dose of 250 r. This smaller inhibitory effect might be accounted for by the fact that the later fractions were administered when the tumours had increased appreciably in size.

The results of the mitotic counts on the cortical region of tumours irradiated with single doses of 250 r and 1000 r are given in Fig. 2. Full mitotic recovery

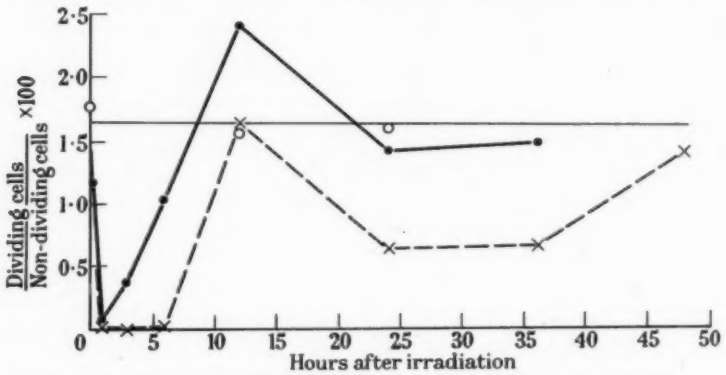


FIG. 2.—Mitotic incidence in the cortical cells of Sarcoma 37 after 250 r and 1000 r.
○——○ Control ●——● 250 r. ×———× 1000 r.

was apparent after about 8 hours in tumours irradiated with 250 r. The subsequent hypermitosis after 12 hours may have been due to a summation effect produced by cells which were in a relatively radio-resistant state during some period of the intermitotic phase. A similar result has been reported by Friedman, Sargent and Drutz (1955) in mitotic counts of the jejunum of rats after irradiation; the peak in this instance occurred at about 8 hours. After irradiation with 1000 r full mitotic recovery was reached after about 48 hours, but the number of viable cells in the cortical region was greatly reduced. Lymphocytes and non-viable tumour cells were present in up to 90 per cent of the cortical region after 24 hours; few viable tumour cells were present 72 hours after irradiation.

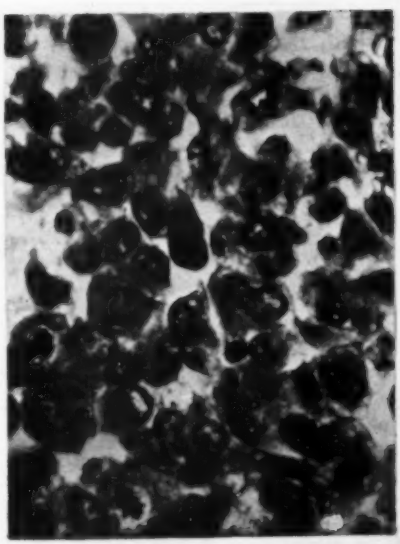
DISCUSSION

From the histological observations, it appeared that the viability of tumour cells irradiated with a single dose of 250 r was not affected. The effect of this dose on the growth rate of this tumour would therefore be a period of delay corresponding to the period of mitotic inhibition. This delay of about 8 hours is

EXPLANATION OF PLATE

Cortical region of Sarcoma 37. Fixed in Susa and stained with haematoxylin and eosin.

FIG. 3.—Control tumour fixed 24 hours after commencement of experiment. × 250.
FIG. 4.—24 hours after irradiation with 250 r. × 250.
FIG. 5.—24 hours after irradiation with 1000 r. × 250.
FIG. 6.—72 hours after irradiation with 1000 r. × 250.



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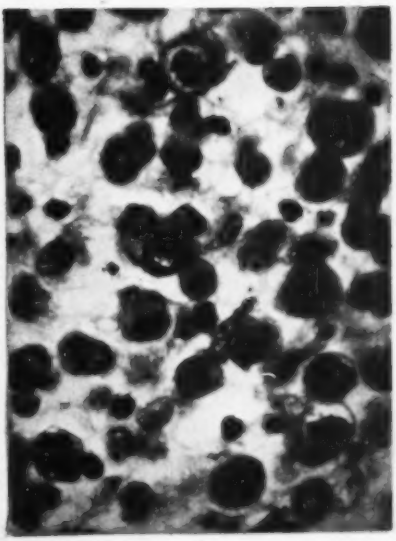

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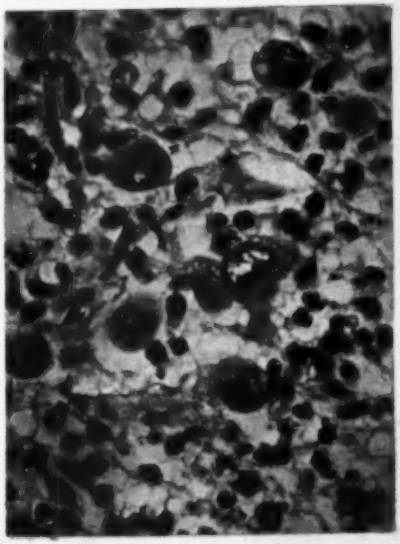

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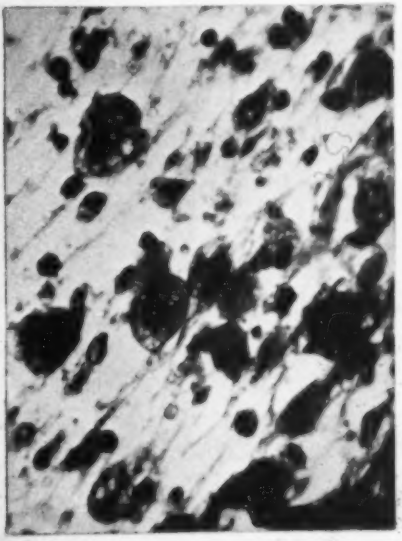

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barely detectable and the growth rate approaches that of the control tumours (Fig. 1). A summation effect, based on the period of mitotic delay, with four fractions of 250 r at 6-hour intervals would give a delay in tumour growth of 24 hours or less over the control growth rate. The delay in growth, however, is of the order of 6 to 7 days. It would appear that under these conditions the important factor is the recovery period after irradiation which affects the viability of the cell. A considerable proportion of the cortex of tumours irradiated at 12-hour intervals, and fixed 48 hours after the last irradiation, consisted of dead cells. This period of recovery was greater than 12 hours after irradiation, although some recovery took place at irradiation intervals of 15 minutes to 12 hours as the summation of these doses was not complete.

This recovery period might be allied to structural damage of the cell with subsequent repair—in which event the fractionation of a given dose of irradiation might not produce an effect greater than the total dose (super-summation). This concept agrees with previous data on fractionation (with the exception of Fogg and Cowing (1953)), where no effects greater than the single total dose were shown. If the recovery period was concerned with damage during some mitotic or intermitotic phase, converting the cell to a postmitotic condition, a suitable choice of time interval and dose might produce a super-summation effect.

SUMMARY

1. The effects of fractionated doses of X-irradiation on the growth of Sarcoma 37 in RIII mice have been studied.

2. With time-intervals of 15 minutes, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours, no increased inhibitory effect over the total dose was observed.

3. Fractionations at time-intervals of from 15 minutes to 12 hours produced comparable growth inhibition.

4. The effects of the fractionation treatments were not related solely to periods of mitotic delay.

5. The action of fractionation treatments in relation to a recovery period is discussed.

My thanks are due to Mr. F. W. Butcher and Mrs. C. E. Warren for assistance with the animal experiments.

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OBSERVATIONS ON HORMONE-DEPENDENT RENAL TUMOURS IN THE GOLDEN HAMSTER

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In a previous paper Horning (1954) reported the hormonal factors determining the successful transplantation of stilboestrol-induced renal tumours in the male golden hamster, the development of which was first described by Kirkman and Bacon (1949). Since then, these experiments on transplantation have been extended to provide additional evidence that renal neoplasia in the hamster comes under the category of hormonal cancer. The results are discussed herein in the light of our present knowledge of endocrine neoplasia in other species of laboratory animals.

MATERIAL AND METHODS

Male golden hamsters of indeterminate ancestry bred in this Institute were used exclusively in these experiments. Primary kidney tumours were induced by implanting 20 mg. pellets of diethyl-stilboestrol subcutaneously in each animal. Each pellet was made from the pure synthetic hormone. They were moulded under pressure, and no binding medium was used. Each tablet was weighed prior to implantation, and each hamster received a second stilboestrol pellet 12 weeks after the first had been implanted. The period of induction between pellet implantation and the development of palpable kidney tumours was considerably reduced following treatment with the second tablet and the incidence of kidney tumours was also increased. Approximately 70 to 80 per cent of all male hamsters develop palpable renal lesions within 9 to 12 months following treatment with a single 20 mg. pellet, whereas the incidence following the introduction of the second is in some instances increased to 100 per cent. It is preferable, although not absolutely necessary, to select hamsters 6 to 8 weeks for this purpose.

The most satisfactory way of obtaining successful transplantable renal tumours was to graft them subcutaneously into host animals which have been pre-treated with a 20 mg. tablet of stilboestrol 3 months prior to receiving the tumour implant. In another separate series of experiments both the stilboestrol pellet and the tumour were grafted simultaneously into normal hamsters which had not been previously treated with the synthetic hormone. The differences in the growth rates between the tumour grafts in these two series of experiments were then later compared.

Tumours were also grafted into the subcutaneous tissues of the tail as well as into the flanks of pre-treated male hamsters. Selected pieces of the kidney tumour were finely minced up in saline, and approximately 0·2 ml. of this suspension was injected. The tail of the hamster is very small, and must be held firmly on a thick cork mat. The point of the syringe needle is then directed towards the tip of the tail, taking precaution to avoid the caudal vein.

All tumours transplanted into oestrogenised hamsters undergo long latent periods in the subcutaneous tissues of their hosts before the development of palpable lesions. The periods of time these tumours remain in the quiescent phase, before active growth commences, is diminished during each successive serial generation of transplants.

Histological studies were made of some of these dormant grafts from the 3rd generation of serial transplants. With the aid of a binocular microscope, quiescent transplants can, with care, be removed from their subcutaneous sites and transferred to fixative without much damage.

Induced renal primary tumours grow as an ascites in the peritoneum of hamsters providing they are transferred into hosts which have previously been oestrogenised. The ascitic kidney tumours were transplanted in 3 serial generations. Material for the 1st transfer was obtained from a hamster with very large multifocal kidney tumours possessing metastatic implants on the abdominal parietes and viscera. Selected pieces of the primary lesion and the implants were finely minced up with saline solution and 0·2 ml. of this suspension was injected intraperitoneally into hamsters all of which had been treated subcutaneously with a 20 mg. pellet 3 months beforehand. At the same time an equal number of hamsters which had not been pre-treated with the synthetic hormone received similar amounts of the saline tumour emulsion intraperitoneally to form the control group.

After a latent period of 6 to 9 months following transfer, the abdomens of 25 per cent of the treated animals became distended with fluid, and smears of this abdominal fluid were prepared for cytological examination. At post mortem, after all the peritoneal fluid had been drawn off, the numerous tumour implants which had grown on the peritoneum, were fixed for histological study.

A study was also undertaken to ascertain if any differences existed between the absorption rates of the subcutaneously implanted stilboestrol pellets when transplanted under identical conditions in male hamsters, albino and desert rats. Twenty mg. tablets of pure stilboestrol were implanted subcutaneously into the left flanks of the three different species of male rodents, each animal being 12 weeks of age at the time of implantation. The implanted hormone tablets were recovered from the rodents at intervals ranging from 7 to 45 days. They were then dried and weighed, and the absorption over a given period was worked out separately for each species of rodent.

For routine histological study, kidney tumour material was fixed either in aqueous or alcoholic Bouin and subsequently stained in either haematoxylin and eosin or by a modification of Masson's light green stain. Pituitary glands were fixed in Zenker-formol and differentially stained by a modification of Mallory's triple stain.

Observations

Endocrine factors involved in successful tumour transplantation

Since the macroscopic appearance and methods of induction of primary kidney tumours in the hamster by oestrogen have been previously reported by Kirkman and Bacon (1949) and by Horning and Whittick (1954), this communication will be concerned mainly with factors controlling the successful transplantation of the tumours. However, for the sake of convenience the macroscopic appearance of a typical primary multifocal and bilateral renal carcinoma is illustrated in

Fig. 1. The majority of kidney tumours were induced 10½ months following the subcutaneous implantation of a 20 mg. stilboestrol pellet, which was followed by a second similar tablet 3 months after the first had been implanted (Fig. 2).

Many attempts have been made without success to graft these induced primary renal tumours in host hamsters. Tumour transplants failed to grow, however, either when grafted subcutaneously or intraperitoneally into intact untreated hamsters of both sexes and of varying ages (Horning, 1954). In several instances metastatic implants from the diaphragm were also transplanted but in every instance these also failed to grow. The untreated hamsters bearing the tumour grafts were kept alive for a year, and were subsequently killed as no palpable lesions had developed. Many animals with negative grafts were killed at intervals varying from 4 to 12 months after transplantation. In every instance both the tumours grafted subcutaneously and those transferred intraperitoneally had been totally absorbed by the host-bearing animals.

Since these primary kidney carcinomas possessed all the histological characteristics of malignant growths and metastasised in many instances via the lymphatic pathway (Horning, 1956), their failure to grow either as subcutaneous grafts or intraperitoneal transfers was surprising.

Consideration was then given to the fact that as the kidney tumours are dependent upon high levels of estrogen for their induction they might also be dependent upon the continuous stimulation of oestrogen in excessive amounts for their maintenance as transplants in host animals. Selected pieces of a large primary kidney tumour were grafted into male hamsters all of which had been previously treated for 3 months with a 20 mg. pellet of stilboestrol. An equal number of normal untreated males likewise received subcutaneous grafts from the same kidney tumour, and these acted as controls. Small palpable nodules began to develop 7 to 8 months in 45 per cent of the stilboestrol-treated hosts, whilst none developed in the untreated group. The experiments were repeated several times with the same results and conclusively demonstrated that these hamster renal tumours are not autonomus lesions since they are dependent upon oestrogen for both their induction and maintenance as grafts (Fig. 3).

The results of grafting the kidney tumours into the subcutaneous tissues of the tails in pre-treated hamsters (Fig. 8) showed conclusively that a higher percentage of blood-borne metastases developed, specially in the lymph nodes, than normally occurs when tumours are implanted into the trunk.

Experiments are being undertaken on the regression of these transplanted tumours following the withdrawal of the hormone pellet. These observations are easier to determine when growing in the tail than they are in the trunk.

Histological examination of the first generation of tumour transplants growing in pre-treated hosts showed them to be actively growing clear-celled carcinomas (Horning, 1954). Although these kidney tumour grafts are now in their 8th generation of serial grafts they still show no evidence of anaplasia (Fig. 7). Also 95 to 100 per cent of the 8th generation continue to grow provided they are transplanted into pre-treated hosts.

Microscopic examination of the ascitic tumours which had grown on the peritoneum, and surfaces of the kidney and other organs, were also similar in their histology to the primary lesion from which the tumour suspension was made. Ascitic tumour deposits on the diaphragm as well as on the renal capsule are seen in Fig. 4. Marcoscopically the lesions have the appearance of early primary

cortical growths, but subsequent histological examination showed them to be only colonies of tumour ascitic cells growing in compact formation and not early primary renal tumours. The tumour implants developed from clumps of ascitic cells which first adhered to the renal capsule and subsequently multiplied, thus forming foci of solid tumours (Fig. 5). Microscopic examination of both kidneys failed to reveal the presence of any primary renal lesions. Another interesting feature was that the peritoneal fluid of these hamsters contained remarkably few ascitic tumour cells. Apart from the kidney tumour cells the fluid contained some detached mesothelial cells and leucocytes. Smears of the peritoneal fluid showed that the ascitic tumour cells were invariably seen in small clumps. Although freed from their complicated stroma and growing in a new medium they were easily recognised. They possessed large nuclei with little chromatin, with a faintly staining cytoplasm, and resembled the clear-celled carcinoma elements which composed the solid tumours (Fig. 6).

When growing as an ascites tumour, the renal carcinomas behave in much the same way as the subcutaneous solid tumours. Following transfer into the peritoneal fluid of pre-treated host animals, they undergo long dormant periods before they begin to multiply and finally enter the malignant phase. But compared with the solid tumours, approximately only 30 per cent of the ascites tumours had grown following transfer.

A study of the histogram (Fig. 9) of subcutaneous transplanted tumours shows that this latent period preceding growth of the graft is gradually reduced following each successive generation of transplants. For instance, in the 1st generation of transplants in one particular line of kidney tumour grafts (Fig. 9) the cells of the transplants remained in the quiescent state for nearly $11\frac{1}{2}$ months before they exhibited any sign of active growth. The 4th generation of renal grafts showed that the dormant period was considerably reduced and that palpable lesions developed 6 to 7 months after grafting, whereas in the 6th generation palpable tumours occurred as early as 2 months following implantation.

One strain of the ascites kidney tumour is now in its 3rd generation of transfer. The 1st generation remained dormant in the peritoneal fluid for nearly 7 months. By this time the abdomens of the host animals became distended with fluid and had to be sacrificed. The 2nd generation took 5 months, and the 3rd $4\frac{1}{2}$ months, before abdominal distention occurred.

Although the ascitic tumour cells in their 1st and 2nd generations of transfer do not remain in the quiescent phase for such long periods of time as the solid kidney grafted tumours, it is of interest to note that in each case the dormant phase of the transplanted cell growing in the peritoneal fluid is likewise gradually reduced during each successive generation.

The histology of the kidney tumour cells growing as solid tumours has been studied during their quiescent phase. Their morphology during the dormant state contrasts greatly when compared with those tumour cells which have entered into the malignant phase. The cells have a dehydrated appearance, are irregular in outline, and both the cytoplasm and the nuclei stain darkly. In many instances it is difficult to differentiate between the shrunken deeply stained nuclei and the cytoplasm. The cells composing a transplant which had suddenly commenced to grow, differed greatly when compared to those forming a dormant graft. The cells were spherical in outline and both the cytoplasm and the nuclei were only faintly stained ; also numerous cells are seen in division.

The structure and behaviour of the ascitic tumour cells during their dormant phase in the peritoneal fluid, owing to obvious technical difficulties, have not yet been studied.

Comparison of absorption rates of subcutaneously implanted stilboestrol pellets in male hamster, albino and desert rats

The fact that hamsters can tolerate very large doses of both the naturally-occurring and the synthetic oestrogens is also of interest. Comparative experiments were therefore undertaken to ascertain the differences, if any, between the rates of absorption of subcutaneously transplanted stilboestrol pellets in the male hamster, and in albino and desert rats. The implanted tablets of pure stilboestrol were recovered from each species of rodent at the same time, at intervals ranging from 7 to 45 days. After the pellets were dried and weighed following various intervals of recovery, a considerable difference between the loss of weight in the tablets recovered from each of the species was clearly noticeable. The rate of absorption of the pellets was found to be considerably slower in the hamster than in either the albino rat or the desert rat (Fig. 10).

In the hamster approximately 24 per cent of the pure stilboestrol pellet was absorbed after 42 days implantation, whilst in the desert rat 45 per cent was absorbed and in the albino rat 55 per cent. After the tablets had been subcutaneously implanted for a period of 14 days it was observed that a marked decrease occurred in the absorption rate of the pellets in each of the three different species of rodent (Fig. 10). It was of interest to note that this deflection occurred at a time when each of the implanted pellets had become encapsulated in the subcutaneous tissues of the host animals.

DISCUSSION

The results of these experiments show conclusively that kidney tumours in the male golden hamster are dependent upon excessive oestrogenic stimulation

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EXPLANATION OF PLATES

FIG. 1.—Macroscopic photograph of multifocal and bilateral kidney tumours in a male golden hamster following 10½ months treatment with two 20 mg. pellets of pure stilboestrol. × 1·7.

FIG. 2.—Macroscopic photograph showing a stilboestrol-induced multifocal renal tumour in one kidney, together with two stilboestrol pellets (indicated by arrows) implanted in the subcutaneous tissues of the host. The smaller of the two tablets, the first to be transplanted, had been implanted for six months and the second pellet for three months. × 1·9.

FIG. 3.—Hormone-dependent kidney tumour growing subcutaneously in a male hamster which had been pre-treated with stilboestrol. The two arrows indicate the hormone pellets. × 1.

FIG. 4.—Macroscopic photograph of a male hamster pre-treated with stilboestrol in which the kidney tumour had been growing as an ascites. Note the tumour implants on the diaphragm, as well as on the capsule of the kidney (see arrow). × 1·7. Compare with Fig. 5.

FIG. 5.—Section of the tumour implant indicated by an arrow in Fig. 4. The tumour had been growing as an ascites and formed a colony on the capsule of the kidney. Macroscopically these lesions growing on the surface of the kidney have the appearance of primary tumours. × 720.

FIG. 6.—Smear preparation made from abdominal fluid of a hamster bearing a kidney tumour as an ascites. Observe the cluster of tumour cells. × 720.

FIG. 7.—Section of a generation solid tumour transplant. This tumour is a clear-cell carcinoma and has not undergone anaplastic changes. × 130.

FIG. 8.—Kidney tumour graft growing in tail of a stilboestrol pre-treated male hamster × 1·3.

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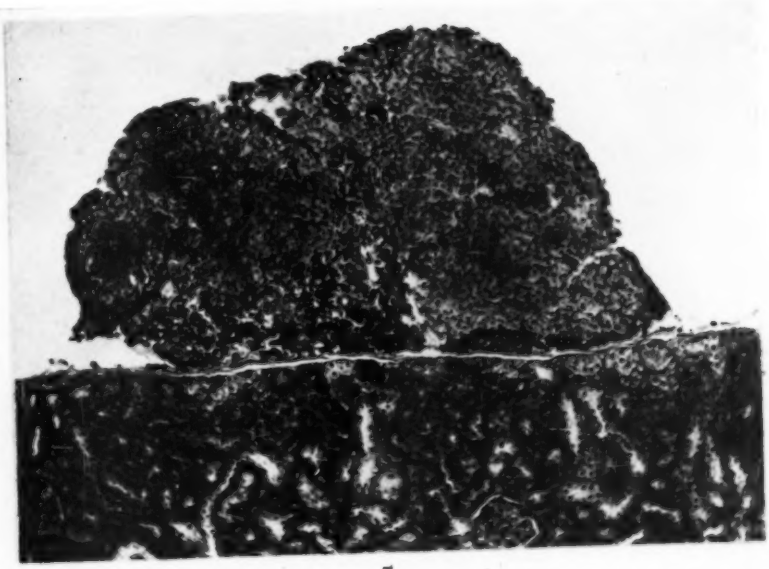

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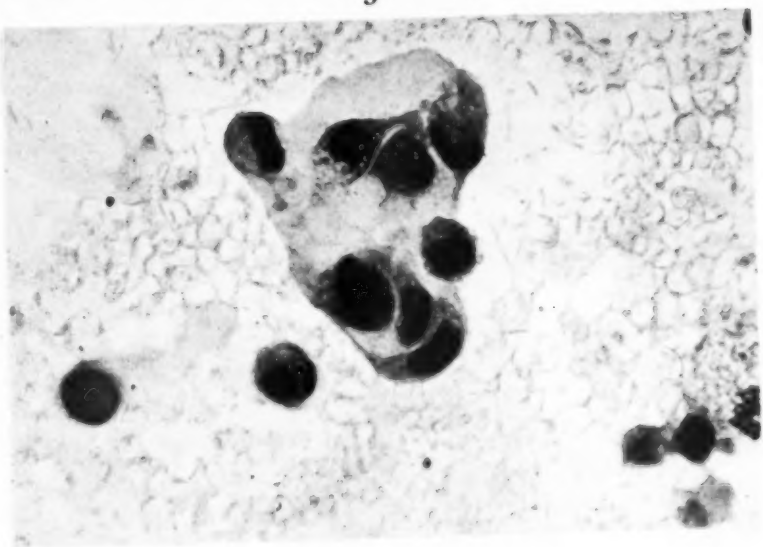

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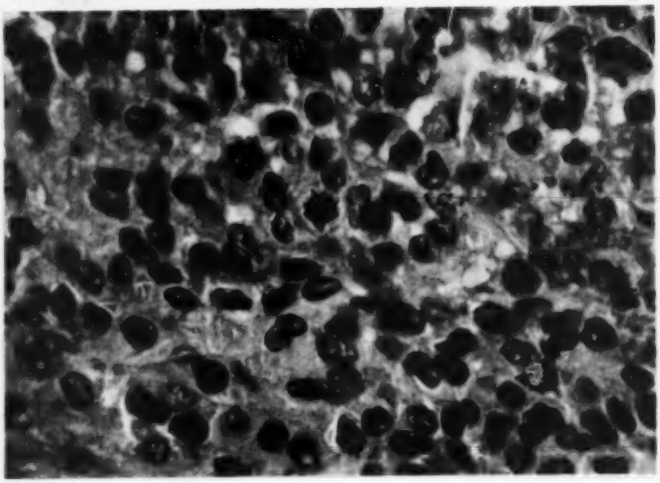

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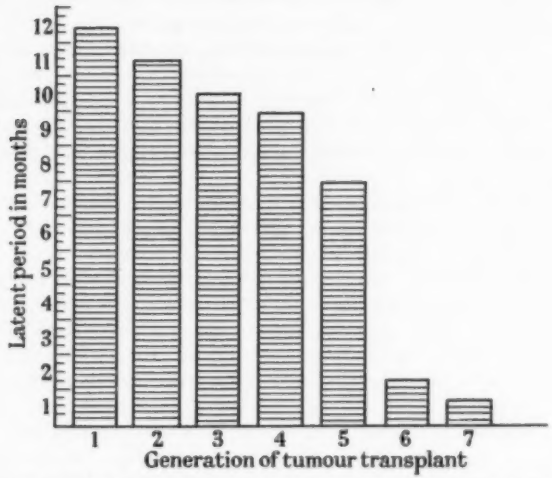

FIG. 9.—Histogram illustrating the decline in the dormant period which kidney transplants undergo in host hamsters prior to the development of palpable lesions. The latent period of tumour growth which was nearly 12 months in the 1st generation has been reduced to one month in the 7th serial generation.

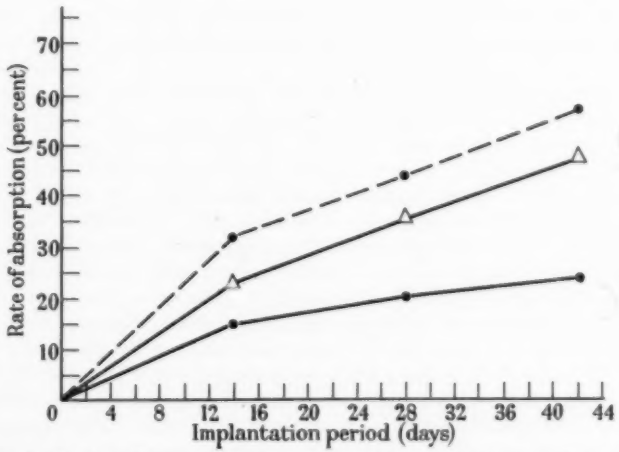

FIG. 10.—Graph showing differences in the rates of absorption of pure pellets of diethylstilboestrol in the golden hamster, desert rat and albino rat. All hormone pellets were implanted subcutaneously.

●——● Golden hamster.
△——△ Desert rat.
●- - -● Albino rat.

not only for their induction but also for their maintenance as transplants in host animals. Because the tumour grafts will only grow in hamsters which have been pre-treated or at the time of their transplantation simultaneously receive oestrogen, they therefore come under the category of endocrine neoplasia. This is further supported by the fact that the development of renal neoplasia in the male hamster can be prevented by early treatment with a hormone antagonist, namely testosterone propionate (Horning, 1956), which neutralises the neoplasm-inducing properties of the oestrogen.

When considering the question of the induction of renal carcinoma in the hamster in relation to the problem of endocrine carcinogenesis, there are several interesting issues to be considered. For instance, tumours which are induced by hormones, and are dependent upon a particular hormone for their sustained growth, invariably develop in organs of the body which belong to the endocrine system or else come under the direct influence of the anterior lobe of the pituitary gland. The kidney, except as a part of the body subject to the stimulation of somatotrophin, is not directly influenced by the pars anterior. Because the kidney is controlled by the posterior lobe which secretes the anti-diuretic hormone regulating the water excretion of the organ, these oestrogen-induced lesions are of exceptional interest.

The rôle played by the pituitary gland in hormone-dependent tumours is important because there are many forms of endocrine neoplasms which fail to develop or else regress following hypophysectomy. The functional dependency of this form of cancer upon the pars anterior was first demonstrated by Korteweg and Thomas (1939), Lacassagne and Chamorro (1939) and later by Gardner (1942).

Agate (1955) on the other hand, has demonstrated that malignant tumours induced by carcinogenic hydrocarbons, which are not hormone-dependent lesions, grow readily in hypophysectomised rodents. These results are further supported by the clinical observations of Huggins (1952) who finds that the growth of skin cancers and other non-endocrine tumours are uninfluenced by total adrenalectomy, which invariably retards the growth rate of hormone-dependent lesions.

Experiments are already in progress in which radio-active seeds are implanted into the pituitary of living hamsters, with the object of destroying the gland, before treating with stilboestrol. By this method it should be possible to observe whether or not induction of kidney tumours can occur in the absence of a functioning pituitary. This would help us to ascertain whether oestrogen acts indirectly through the pituitary or whether the effect is a direct one upon the kidney itself.

There is a tendency for some rodent hormone-dependent tumours gradually to lose their dependence upon a particular hormone following several serial generations of grafting in host animals (Foulds, 1947 ; Horning, 1949). This, however, is not always the case for Mühlbock (1954, personal communication) has recently found that some ovarian tumours induced by the action of pituitary gonadotrophins, which have been growing as serial transplants for over two years, still remain dependent upon hormonal administration for their continued growth. They fail to grow when grafted into untreated animals. In a similar manner, this particular line of kidney tumour transplants has been growing as a serial graft for nearly four years, and still retains its dependence upon oestrogen.

Other experiments by Gardner (1954) and Mühlbock (1953) have a direct bearing on the results obtained by tumour transplantation in the hamster. The former investigator has found that oestrogen induced pituitary tumours in mice will only grow when transplanted subcutaneously into oestrogen-treated

hosts but not in untreated mice. Mühlbock also reports results which are similar to those of Gardner, in that oestrogen-induced pituitary tumours will only grow when grafted into mice which have themselves already developed spontaneous hypophyseal lesions.

Bielschowsky *et al.* (1949) induced thyroid tumours in rats by treatment with methylthiouracil, which like the renal carcinomas in the hamster, possess all the histological characteristics of malignant lesions, and yet will not grow when grafted into normal healthy rats. These workers, however, later discovered that the thyroid tumours would grow if grafted into rats already in a state of thyroid deficiency. The fact that these transplanted tumours are dependent for growth upon an increased output of thyrotrophic hormone in host-bearing rats is of interest and demonstrates that these particular thyroid neoplasms of Bielschowsky, like the pituitary tumours of Gardner and Mühlbock and the renal tumours in the hamster, cannot be considered as autonomous lesions.

The reasons why some tumours from their induction are hormone-dependent for their sustained growth and later become hormone-independent are not fully understood, although this process is observed both clinically and in laboratory animals during serial transplantation. Nor is it understood why some lesions, which arise in identical organs under endocrine influence, are from their earliest development resistant to any form of hormonal modification, whilst others rapidly respond to treatment.

When a tumour becomes an autonomous lesion it is composed of permanently altered cells and is freed from its sensitivity to the hormonal forces which control the cells of endocrine-dependent tumours. It is quite conceivable as Furth (1955) contends, that some malignant growths might be composed of both dependent and autonomous cellular components, and this might explain why a hormone-dependent tumour whose growth has previously been inhibited by means of endocrine therapy, will suddenly lose its responsiveness to a particular hormone, and become an uncontrolled autonomous neoplasm.

It therefore cannot be assumed that all oestrogen-induced kidney tumours in the hamster will necessarily be endocrine-dependent lesions, but up to the present no hormone-independent kidney tumour has developed amongst the treated hamsters in these laboratories.

Mühlbock (1956, personal communication) for instance induced 19 different granulosa-celled tumours in young mice, and out of this number 15 were dependent upon either oestrogen or androgen for their sustained growths as grafts in host mice of the same strain. Although the majority of these tumours were hormone-dependent lesions, it is interesting to note that hormone-dependent neoplasms can in some instances, be induced by agents other than hormones. Thus Van Nie and Mühlbock (1956) induced hormone-dependent ovarian tumours by irradiating infantile mice, and Horning (1949) induced an androgen-dependent prostatic adenocarcinoma in mice by treatment with a carcinogenic hydrocarbon.

Other striking aspects of the behaviour of grafted renal tumours, are the long dormant period which exists between transplantation and the appearance of palpable lesions, and the fact that the periods of time during which the tumour cells remain in the quiescent state is gradually diminished during each successive serial generation of transplants. Gardner (1954) has also studied the dormant state in transplants of oestrogen-induced testicular tumours in pure line mice. These neoplasms, like those of the hamster, were hormone dependent and only

grew when grafted in oestrogen-treated hosts of the same strain in which they were induced. They remained in the quiescent phase for periods of over 7 months before commencing to grow. Furthermore, the transplants regressed when the hormonal stimulus was withheld. Gardner further found that the testicular tumour cells when in the dormant state of their existence also differed considerably in their histology from the cells observed in the proliferating phase. Like Gardner, the author also has found a marked morphological difference between the quiescent kidney transplanted component and the proliferating cell. Gardner (1954) interprets the proliferation following long periods of quiescence as being due to the fact that oestrogen produces changes in the function of the anterior pituitary by reducing its gonadotrophic as well as growth-hormone content. Considering the latent period of the grafted kidney tumour cells in the hamster was diminished from eleven and a half months in the first generation before palpable lesions arose to only one month in the 7th serial generation, it would be difficult to attribute the decline in the latent period solely to changes in pituitary function induced by oestrogen treatment.

Franks (1954) has made an exhaustive study of latent carcinoma of the prostate in man. He finds that the prostatic tumour cells are capable of remaining in a dormant state and suggests that oestrogen may be a factor involved either directly or indirectly in inducing active proliferation. This author has also detected small latent tumours in human post-mortem material in both the kidney and the thyroid. Unlike the hormone-dependent dormant tumours in laboratory animals, no morphological differences could be distinguished by Franks (1954) between the latent and malignant cell.

The difference in the rates of development of palpable lesions, between kidney tumours grafted into hamsters which have previously been pre-treated with oestrogen and those which receive the tumour transplant and hormone pellet simultaneously is another feature of interest. Tumours when grafted into hamsters which have already been treated with stilboestrol 3 months previous to receiving the graft, develop twice as rapidly as they do in untreated hosts which receive both the graft and the hormone tablet together. This again stresses the dependency of these tumours upon hormonal stimulation, but whether this is dependent upon the function of the pituitary gland is a matter of speculation. Experiments are being undertaken to ascertain if renal grafts will grow in non-oestrogenised hamsters bearing oestrogen-induced pituitary tumour implants, as it would give valuable information on the biological mechanism controlling the behaviour of hormone-dependency.

Baserga and Baum (1955) were the first to demonstrate that tumours, which normally metastasise when grafted into the subcutaneous tissues of the trunk, yield a higher per centage of blood-born secondaries when transplanted into the tail. The results obtained in the present experiments by grafting into the tails of hamsters confirms the findings of these authors, who rightly emphasise the importance of the site of the primary growth in determining the production of metastases. This method should be useful not only in studying the mechanism of metastases, but specially in determining the chemotherapeutic effects of drugs on grafted tumours, since the spread of the neoplasms via the lymphatics is more comparable to conditions which frequently occur in man.

The differences in the absorption rate between the pellets of pure stilboestrol implanted subcutaneously in the hamster, desert rat and albino rat have yielded

some interesting information. As has been shown, the absorption rate of the hormone tablet in the hamster is much slower than it is in the other two rodents, and this might explain why the hamster is able to tolerate doses of oestrogen better than most rodents do. The deflection in the absorption rate of the tablets in each of three species of rodent occurs approximately on the 14th day at a period when the pellets become encapsulated in the subcutaneous tissues of the host. Cowie and Folley (1946) have shown, however, that this sudden falling off is due solely to a marked decrease in the surface area of the implanted pellets and not to the influence of encapsulation.

SUMMARY

(1) Hormonal factors regulating the growth of transplanted stilboestrol-induced renal tumours in the male golden hamster are discussed.

(2) The appearance and behaviour of the dormant phase of grafted kidney tumours which exists in oestrogenised host hamsters prior to the development of palpable lesions has been described.

(3) Kidney tumours grafted subcutaneously into the tails of pre-treated hamsters metastasise more readily than those grafted into the subcutaneous tissues of the trunk.

(4) Comparison between the absorption rates of subcutaneously implanted pellets of pure stilboestrol in the hamster, desert rat and albino rat, shows that the absorption rate of the hormone pellet is slower in the hamster then it is in the other two species of rodents.

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A NEW STRAIN OF MICE WITH A HIGH INCIDENCE OF MAMMARY CANCERS AND ENLARGEMENT OF THE PITUITARY

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THE beneficial effects obtained by hypophysectomy in the treatment of advanced cancer of the human breast have revived interest in the role of pituitary factors in the pathogenesis of mammary tumours (Coppedge and Segaloff, 1951; Segaloff, Gordon, Horwitt, Schlosser and Murison, 1951; Scowen and Hadfield, 1955; Hadfield, 1956*a* and *b*). Many years ago experimentalists produced evidence for a promoting influence of the adenohypophysis on the growth of mammary cancers in mice. Morphological descriptions of the pituitary in this condition remain scanty and to our knowledge no pattern has emerged which could be called typical for cancer of the breast. When we came into the possession of a high mammary cancer strain, the pituitaries of which showed gross abnormalities, it was hoped that a useful tool had become available for a reinvestigation of the role of pituitary hormones in the development of tumours of the breast. In the following an account will be given of the origin of this strain (NZY), of the endocrine abnormalities observed in the females and of the neoplasms occurring in animals of both sexes.

METHODS AND MATERIAL

The mice were kept in thermostatically controlled rooms at a temperature $70 \pm 2°$ F. The composition of the stock diet as well as of the food given to the breeders has been described in a previous communication (Bielschowsky and Bielschowsky, 1956).

At the age of 28 days the animals were separated from their mothers and the female offspring from the male. Some of the females were kept as virgins, the others, at the age of 8 weeks, were mated to their brothers with whom they remained until they had produced and reared 4–7 litters. However, when a breeder failed to become pregnant during a period of 3 months the animal was removed from the breeding box.

Forty males, 31 virgins and 84 breeders belonging to the 18th–24th generation were kept under observation during their full life span. They were killed when they appeared to be seriously ill or when palpable tumours were discovered. Some mice with mammary cancers were allowed to live for 5–8 weeks after the tumour was first observed. It was impossible to keep a constant number of animals per box but care was taken to avoid overcrowding. The maximum number of mice at any one time never exceeded 15 in boxes measuring $28 \times 43 \times 13$ cm.

The material presented in this paper consists of the 155 mice mentioned above together with 43 healthy females and 6 males killed for a study of the endocrine glands and their target organs.

Tissues taken for histological study were fixed in Helly's fluid or in formalin-saline. The sections were stained with haematoxylin-eosin. The following special staining techniques were used: Lillie's oil red O method for the demonstration of lipids; the procedure of Tirmann and Schmelzer as given by Romeis (1948) for iron pigment; Lillie's allochrome or the van Gieson method for connective tissues.

The pituitaries were placed immediately after removal into weighed bottles containing 2 ml. of sublimate-formalin and stained by the methods given in a previous paper (Bielschowsky, 1953).

RESULTS

The origin of the strain

The strain designated NZY is now in the 29th generation. It originated in 1948 from a pair of mice with tan coat colour selected from the mixed colony kept in the Animal Department of the Medical School. One of the males of the 2nd generation of brother-sister mating was a piebald. He was mated to a sister with the original coat colour. In the 4th generation the first piebald female became available and was mated to a spotted brother. From that time only animals showing this character were used for propagation with the result that in the 7th and subsequent generations only piebald mice were born. Breast cancers appeared in breeding females of F10 and became more frequent in later generations. In the mixed mouse colony of the Medical School which maintains about 500 breeding females throughout the year breast cancers have been seen only twice during the last 5 years. However, most of the breeders are killed when less than 12 months old and nothing is known of the incidence of spontaneous tumours in older animals. Breast cancers do not occur in appreciable numbers in the other 4 inbred strains developed by us concurrently with the NZY. In fact, although many females completed their full life span, only one case of spontaneous mammary cancer has been found so far in the NZO and another in the NZC mice. A few appeared in old females of F14–15 but none in the following generations of the third strain; in the fourth this type of tumour has never been observed.

When complete autopsies of NZY mice were routinely performed it soon became evident that the great majority of females of the 18th and following generations had pituitaries which were either symmetrically or irregularly enlarged. We do not know when this tendency for pituitary enlargement appeared, it was first noticed in the 11th generation.

Some anatomical and physiological features

Adult mice reach relatively high weights without being obese. The maximum weight of males is 42 g., that of breeding females 40 g. and of virgins 38 g. Values 3–5 g. lower are more common in our material and correspond to the usual weights of adult mice is the mixed colony from which the NZY mice originated. The animals are pink eyed. The pattern of white spotting varies considerably in extension and location. There is frequently a broad band of white hair encircling the lower back and abdomen. The fur covering the latter is generally of a lighter tan than that of the back. Deformities of the thorax are frequent and this strain appears to be more liable to infections of the lung than the other inbred mice kept in the department. However, losses have been light and the average life span has not been reduced significantly by such infections.

The females reach sexual maturity at an early age. The vagina opens between the 28th and 35th day and some mice become pregnant already at the age of 5 weeks. Vaginal smears taken for periods of 10 days in consecutive months revealed that regular 4–5 days cycles are rare in NZY virgins and that dioestrus lasts often for 7–9 or even more days. It seems, however, doubtful whether vaginal smears provide evidence that can be trusted entirely. We have observed more than once in stained sections of the vagina a squamous keratinizing epithelium covered by a layer of an exudate containing many leucocytes and desquamated cells. A viscous mucus-like fluid containing many polymorphs was frequently seen when taking smears.

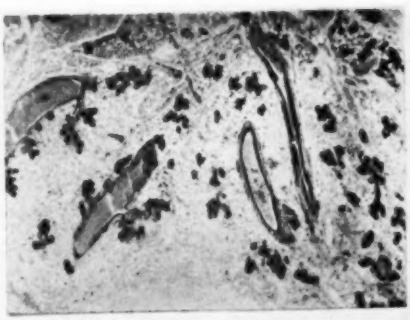
In agreement with these observations is the irregular breeding performance. Some animals do not become pregnant when mated to males of proven fertility whereas others have their litters in rapid succession. Twelve of 91 animals belonging to F20–24 failed to become pregnant. Of the 79 fertile females 56 had a second, 29 a third and 21 a fourth litter. The number of new born mice in 62 first litters was 264. Of these 204 reached weaning age and the same rate of survival was found in second, third and fourth litters. The average litter-size was 4·2 for the first and slightly lower for subsequent pregnancies. It has to be stressed, however, that litter-size ranged from 1–12. The interval between the birth of two consecutive litters varied between 19 and 90 days. The females have a well-developed maternal instinct and even virgins 18 months of age will build a nest and care for newborn given to them.

The sex organs and adrenals

As already mentioned long periods of dioestrus occur frequently in young sexually mature females. In addition mucification of the vaginal epithelium was

EXPLANATION OF PLATES

FIG. 1.—Breast gland of virgin 3 months old. H. & E. × 18.
FIG. 2.—Breast gland of virgin 11 months old. H. & E. × 18.
FIG. 3.—Breast gland of virgin 20 months old. H. & E. × 18.
FIG. 4.—Macroscopically recognizable ducts. Breeder 16 months old.
FIG. 5.—Early intra-alveolar neoplastic lesion. Breeder 11 months old. H. & E. × 18.
FIG. 6.—Early intra-alveolar neoplastic lesion. Breeder 15 months old. H. & E. × 18.
FIG. 7.—Area of squamous metaplasia. Virgin 13 months old. H. & E. × 80.
FIG. 8.—Early adenoacanthomatous lesion. Breeder 15½ months old. H. & E. × 80.
FIG. 9—Keratin cyst from nipple area showing connection of the cyst with a mammary duct. Virgin 20 months old. H. & E. × 18.
FIG. 10.—Intratubular carcinoma. Breeder 21 months old. H. & E. × 80.
FIG. 11.—Intraductal papilloma. Breeder 11 months old. H. & E. × 18.
FIG. 12.—Intraductal papilloma. Breeder 21 months old. H. & E. × 80. (The lesions shown in Fig. 11 and 12 were found in breasts free of palpable tumours.)
FIG. 13.—Sebaceous lesion in mammary cancer. Breeder 10 months old. H. & E. ×113.
FIG. 14.—Sebum cells in midst of mammary cancer. Breeder 16 months old. H. & E. × 495.
FIG. 15.—Sebaceous adenoma of the skin. Breeder 17 months old. H. & E. × 27.
FIG. 16.—Sebaceous adenoma of skin in nipple area. Virgin 18 months old. H. & E. × 113.
FIG. 17.—Symmetrical enlargement of pituitary. Pituitary weight 7·0 mg. Virgin 8 months old, stilboestrol treatment for 6 months. Papanicolaou × 30.
FIG. 18.—Asymmetrical spontaneous enlargement of pituitary. Pituitary weight 5·2 mg. Papanicolaou × 30.
FIG. 19.—Typical picture of pituitary tumour. Pituitary weight 39·5 mg. Breeder 16 months old. Papanicolaou × 560.
FIG. 20.—Group of giant acidophils in pituitary tumour. Pituitary weight 3·5 mg. Breeder 11 months old. Papanicolaou × 560.



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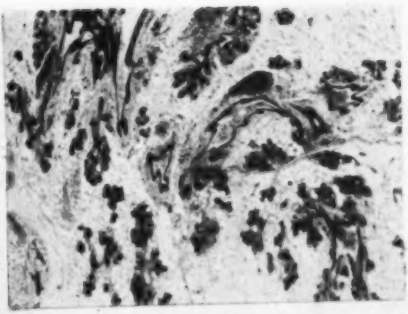

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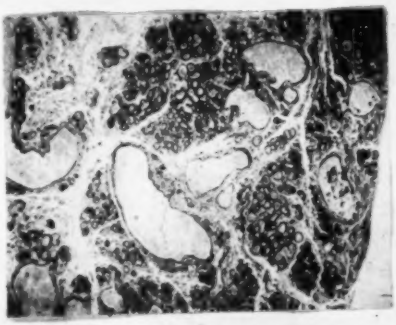

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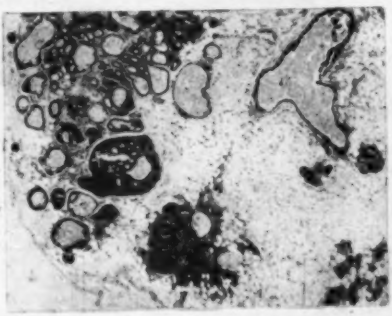

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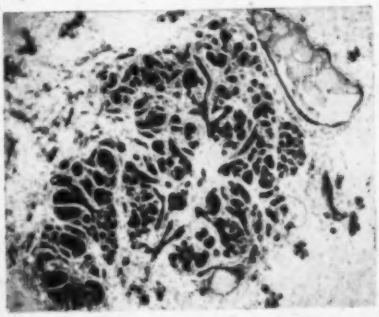

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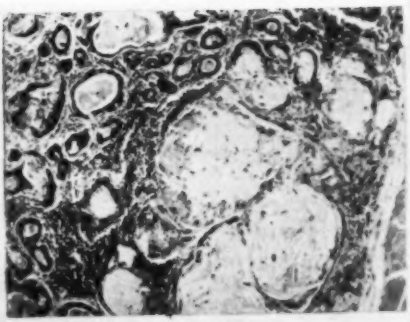

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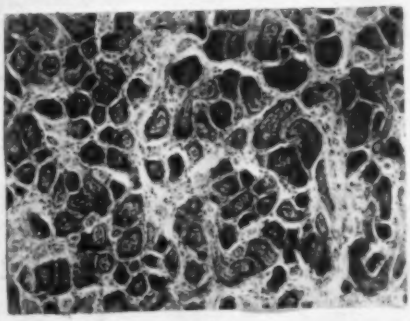

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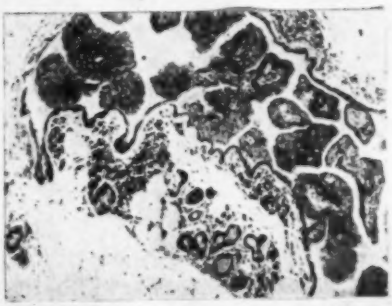

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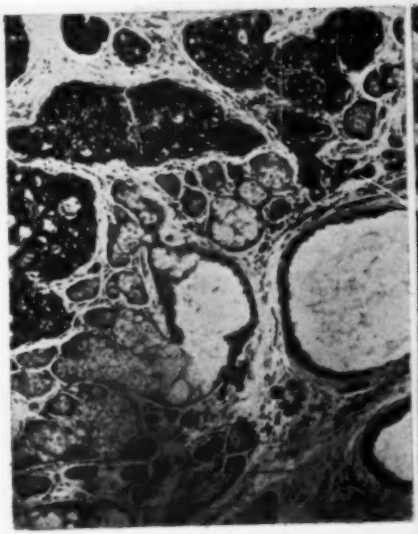

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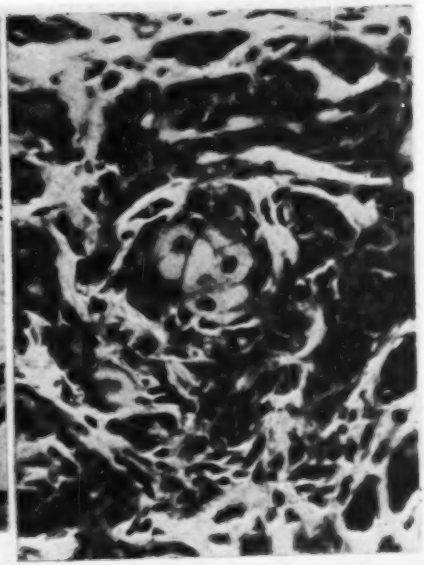

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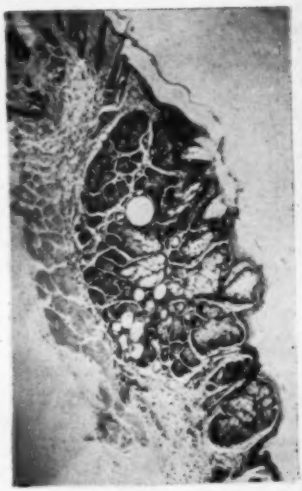

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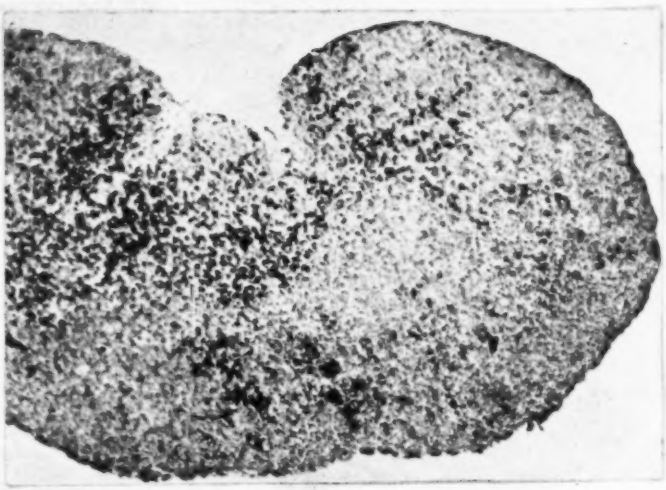

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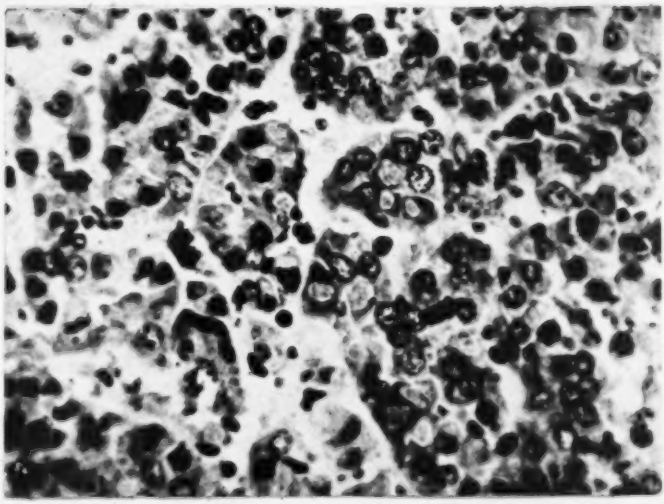

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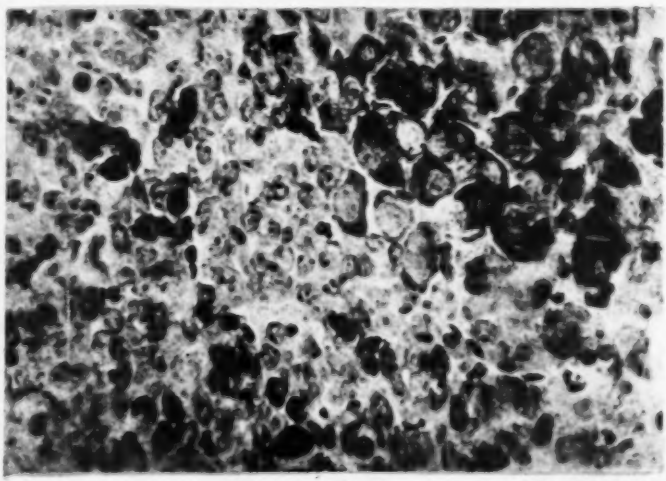

20

Bielschowsky, Bielschowsky and Lindsay.

seen occasionally in stained sections. These observations motivated an inquiry into ovarian function and an attempt was made to induce deciduomas in normal virgins. For this purpose 20 mice, 95–110 days old, were used, 18 being in dioestrus and 2 in oestrus at the time of operation. When these animals were killed 4 days later deciduomas, 2 of macroscopic and one of microscopic size, were found in 3 mice. On histological examination 6 of the 18 animals which had been in dioestrus when operated upon showed marked mucification of the vagina and the deciduomas occurred in 3 of them. These findings indicate the presence of functionally active corpora lutea in unmated mice of the NZY strain. The ovaries contained 2 sets of corpora lutea, 10–12 of these structures being counted in sections from the area of maximum circumference. Large cells were present in many corpora lutea, especially in those from ovaries of mice with deciduomas or with mucification of the vagina. They did not quite reach the size of the lutein cells seen in lactating animals of similar age. The thyroids showed a normal picture, the colloid containing acini were lined by a cuboid epithelium. In the adrenal cortex a well-developed X-zone was seen only once and rests of this structure were still recognizable in a few animals. Small groups of the so-called A-cells of Woolley and Little (1945) were occasionally noted. The breast glands were remarkably well developed for virgins of this age (Fig. 1). There was a considerable variation in the number of alveoli present in the sections, in the degree of secretory activity as well as in the width of the ducts, some of which were definitely dilated.

In order to obtain some information on the endocrine status of females belonging to the age group in which mammary cancers begin to appear and the pituitaries to enlarge, 6 virgins, 6 breeders and 5 stilboestrol-treated females 8–11 months old were killed. The findings were compared with those obtained in 6 spayed animals 12–13 months of age. The ovaries of 11 intact untreated animals resembled those of 3–3½ months old mice, a picture which remains unchanged up to the age of 14 months. They contained follicles in all stages of development and multiple corpora lutea with rather large cells. Dividing lutein cells were often seen in recent corpora lutea. Only one of the virgins had ovaries free from corpora lutea. In this virgin the uterus weighed 185 mg. and the vagina was lined by a thick layer of squamous keratinizing epithelium, whereas in the other mice the weight of the uterus averaged 82 mg. and mucification of varying intensity was frequently present in the epithelial layer of the vagina.

The adrenals also differed little from those of the younger animals. The small subcapsular A-cells were present in larger numbers and reached deeper layers of the cortex. In 3 mice nodules of microscopic size, formed by large elements with foamy cytoplasm resembling the cells of the fascicularis were found in the adrenal cortex.

The mammary glands of the older females could easily be distinguished from those seen at 3–3½ months. Hyperplasia was already recognizable at autopsy. This increase in mammary gland tissue was due mainly to proliferation of alveoli (Fig. 2) which now began to form lobules. Pronounced secretory activity of the mammary epithelium and cystic dilatation of ducts was noted in 6 animals. These signs of enhanced functional activity were more marked in the breeders. Foci of lymphocytes and plasma cells, so-called inflammatory lesions, were present and the connective tissue had increased, especially around the ducts. These findings were common to virgins and mated animals, but in 3 of the breeders

early neoplastic changes were also found. In the breast gland of an 11 months old mouse a small nodule just recognizable by naked eye inspection was noted at autopsy. This was found to be an intraductal papilloma (Fig. 11). In 2 other animals the histological examination revealed the presence of solid alveoli or tubules formed by an atypical mammary epithelium. We shall return to these neoplastic lesions when describing the mammary cancers.

During the second year of life the breast glands of virgins and breeders become increasingly hyperplastic. They are clearly recognizable at post mortem by their darker colour. Small cysts filled with a milky fluid were occasionally seen, but far more frequent were small nodules just visible to the naked eye. They were slightly harder to touch than the surrounding tissue. In 4 old breeders the ducts of one mammary gland were macroscopically recognizable as a black tree against the lighter background (Fig. 4). In other old animals diffuse areas of brownish discoloration were seen.

The first right or left breast gland which could be easily removed *in toto* was always taken for microscopic study in addition to any gland showing macroscopic abnormalities. The histological study of this material gave the impression of a continued growth process which affected ducts, alveoli and, to a lesser extent the connective tissue and did not abate in old age. A typical example of hyperplasia of the breast is depicted in Fig. 3 which shows a mammary gland of a 20-months-old virgin. In this animal the development of the breast had reached a degree comparable to that seen at the end of pregnancy. Functional activity varied from gland to gland and even in parts of the same breast, but in some animals nearly every lobe was secreting. Signs of senile involution were observed only in animals suffering from intercurrent disease.

The blackish discoloration seen at autopsy was found to be due to deposition of large amounts of haemosiderin in the epithelial cells lining the ducts and in macrophages nearby, whereas the brownish material filling the lumen failed to give an iron reaction. Routine sections taken from glands which at autopsy showed the picture of diffuse hyperplasia often revealed the presence of nodules of different kinds. Some consisted of groups of normal and/or atypical alveoli, some were areas of metaplasia and some were undoubtedly neoplastic lesions (Fig. 5, 6). The former have the same appearance as the so-called hyperplastic nodules found in most high breast cancer strains. The metaplastic lesions consisted of groups of alveoli lined by squamous, keratinizing epithelium (Fig. 7). Sometimes only one or two neighbouring alveoli were thus affected but often these lesions were more extensive. Around such squamous foci which commonly occur in virgins and breeders above the age of 14 months, lymphocytes had accumulated in large numbers and these changes were accompanied by an increase in connective tissue. In some of the macroscopically visible nodules similar metaplastic lesions were seen. Here the alveoli had become distended by masses of keratin and in most the adjacent alveoli were lined by an atypical high cuboid epithelium with strongly basophilic cytoplasm ; mitoses were frequent (Fig. 8).

Cystic lesions were found in the nipple area of 8 animals. They were lined by a squamous epithelium and filled with keratin. By serial sectioning it was possible to demonstrate a connection between these cysts and a mammary duct (Fig. 9).

In contrast to the progressive hyperplasia of the breast, the ovaries of aged animals show regressive changes. After the 14th month of life corpora lutea

decrease in numbers, the lutein cells become smaller and finally even inactive corpora lutea are no longer present. Vagina and uterus show either the picture of unopposed oestrogenic stimulation or signs of beginning involution, the latter being more frequent in the very old animals.

Vagina, uterus and mammary glands of 5 of 6 mice ovariectomized at 6 weeks were found to be atrophic 12 months later. The uterus of the sixth weighed 47 mg. as compared with a mean of 16 mg. in the others. The adrenals showed a higher degree of nodular hyperplasia than is commonly seen in intact females of similar age. So far we have not observed in old spayed females hyperplasia of breast glands or mammary tumours.

The mammary cancers

Table I gives the incidence of palpable mammary cancers in females older than 6½ months, the age at which the earliest breast cancer was discovered in a breeder. In virgins no breast tumours were found before the 14th month. Of the 31 unmated females available for study 20 were 13–22 months old when killed and 7 of these had one or more mammary cancers. In 49 of the 84 breeders breast tumours developed. If any conclusions can be drawn from these small numbers, it seems that in virgins breast cancers develop relatively late in life and that their incidence rises with advancing age. In breeders breast cancers develop earlier and the incidence diminishes after the age of 17 months. There was no evidence for an increased frequency of mammary cancers in certain families and no relationship could be established between number of pregnancies and incidence of breast cancers. Of 7 mated animals without offspring 3 had palpable breast tumours and a 4th an early adenoacanthoma. If all the neoplastic lesions had been counted which were discovered either at autopsy where they presented themselves as small nodules, or at microscopic examination of breast glands, the incidence of breast tumours would be considerably higher. By excluding the non-palpable lesions we have eliminated those whose fate was uncertain. In our experience nearly all the palpable tumours grow progressively, although there is good reason to believe that not all the neoplastic lesions progress to malignancy. Regression of a palpable tumour was observed only once. This occurred in a nursing animal.

TABLE I.—*Incidence of Breast Tumours in Virgins and Breeders*

	Virgins			Breeders		
Age group (months)	6–12	12–18	18–22	6–12	12–18	18–23
Total number	11	13	7	21	49	14
Mice with tumours	0	3	4	14	32	3

Of the 56 mice with breast cancers 50 had 1, 5 had 2 and 1 had 3 palpable tumours. These numbers probably do not reflect the true incidence of multiple mammary cancers since most mice were killed soon after a subcutaneous mass was found. In 6 instances the interval between discovery of a breast tumour and autopsy was 5–8 weeks and still no other palpable tumour appeared. Distant metastases were found in 4 animals, three times in the lung and once in the liver.

The morphology of the breast tumours

Of the 63 palpable tumours available for classification 35 had a predominantly alveolar structure (Group A and B of Andervont and Dunn, 1950). In 9 of these

some secretory activity was noted, and in 3 others small foci formed by squamous keratinizing epithelial cells were present. In 7 breast tumours the metaplastic changes were so extensive as to justify the diagnosis of adenoacanthoma. In the remaining 19 carcinomas an alveolar structure could not be found or was only occasionally recognizable. In 8 of these tumours the epithelial cells grew in the form of solid tubules surrounded by a delicate stroma. A well-differentiated specimen of such an intratubular carcinoma (Bonser, 1954) is shown in Fig. 10. Some neoplasms were quite undifferentiated and two, formed by detached cells, resembled closely the tumour depicted by Andervont and Dunn (1950) in their Fig. 6. Two of the breast tumours were sarcomas, one of which contained a few nests of atypical epithelial cells. In 6 animals intraductal papillomas were found (Fig. 11, 12). They occurred in glands not affected by a palpable tumour.

Quite unusual was the presence of sebaceous elements in 4 cancers of the breast. As seen in Fig. 13 islands of hyperplastic sebaceous glands were surrounded by cords of typical mammary cancer cells. Blind ending ducts lined by a stratified squamous epithelium were present but no hair follicles were found in these lesions. In one case it was possible to measure the distance between epidermis and an accumulation of sebaceous glands, it was found to be 2·5 mm. Sebaceous cells were present not only as distinct adenoma-like structures, but twice also in the form of small groups in the midst of mammary cancer cells, as if these elements had differentiated into sebum cells (Fig. 14).

Sebaceous adenomas of the skin

The sebaceous lesions found in sections of otherwise typical breast tumours should be distinguished from sebaceous adenomas of the skin, 6 of which were found in females 12–20 months old. They were situated between epidermis and panniculus carnosus (Fig. 15). All were benign, slow growing and one underwent cystic degeneration. They arose in different areas of the skin, 2 were found near normal nipples (Fig. 16) and 1 in the perineum.

The pituitary

All the 40 untreated males, the oldest of which reached the age of 24 months, had normal pituitaries (maximum weight 2·2 mg.) but in the females pituitary weight varied with age.

In intact females the pituitary weights are normal at the age of $3\frac{1}{2}$ months, increased at the age of 8–11 months; gonadectomy early in life prevents this enlargement of the gland. When a pellet weighing approximately 5 mg. and consisting of 97·5 per cent cholesterol and 2·5 per cent stilboestrol was implanted into normal males or females 4–5 weeks old their pituitaries were found to be grossly enlarged 7–8 months later. This weight increase was most striking in the males. These results are summarized in Table II.

Histologically the adenohypophysis of intact 3–$3\frac{1}{2}$-months-old virgins showed a normal distribution of chromophil and chromophobic elements. At the age of 8–11 months however, the anterior lobes of intact untreated females contained areas, mostly situated near the posterior border and in the most lateral part of the adenohypophysis, in which fully granulated acidophils were reduced in numbers. In these regions a partially or totally degranulated cell with a large Golgi body predominated. Since all transitions from fully granulated acidophils to completely degranulated forms were present we do not doubt that the latter

TABLE II.—*Pituitary Weights of Normal and Spayed Females and of Stilboestrol-treated Animals*

Virgins				Breeders		Spayed female.		Stilboestrol-treated			
								Female.		Male.	
Age (months)	mg.	Age (months)	mg.	Age (months)	mg.	Age (months)	mg..	Age (months)	mg.	Age (months)	mg
3½	1·7	8½	3·4	8	3·2	12	1·3	8½	5·6	8	17·1
3½	1·8	10½	3·4	10	3·2	12	1·3	9	6·0	8½	20·0
3½	2·0	11	2·8	11	3·0	12½	1·6	9	6·3	9	17·8
3½	2·0	11	3·0	11	3·2	12½	2·0	9	7·0	9	18·7
3½	2·1	11	3·1	11	3·4	13	1·3	9	7·2	9	20·2
3½	2·2	11	4·0	11	3·6	13	1·3	—	—	10	22·4

are derived from the acidophilic cells with enlarged Golgi apparatus. A moderate number of mitoses was also found in these areas and it was often possible to identify the dividing cells as acidophils by their granulation. At this stage only a very mild hyperaemia was noticeable so that the gain in weight of the pituitary which takes place between the 4th and 8th month of life must be attributed mainly to an increase in acidophils and chromophobic cells derived from them. These changes were entirely absent from the pituitaries of spayed animals and much exaggerated in those of stilboestrol-treated virgins. A typical example of a symmetrically enlarged pituitary of a stilboestrol-treated female is depicted in Fig. 17. Here nearly half of the anterior lobe was free of normal, fully granulated acidophils. From the results given in Table II it is obvious that pituitary enlargement occurs only in the presence of the ovaries or after treatment with oestrogen. To judge from the atrophy of the secondary sex organs seen one year after ovariectomy, it seems that the amount of estrogen secreted by the adrenals is negligible and that the ovaries are the major source of oestrogen in this strain. The pronounced enlargement of the pituitaries of the males treated with stilboestrol leaves no doubt that in the NZY strain this gland is highly susceptible to oestrogen.

With progressing age the pituitaries of intact, untreated NZY females tend to increase in size and become more hyperaemic. Table III gives the range of pituitary weights of 105 untreated females 6½–24 months old. Only 19 animals had pituitaries weighing less than 3 mg. and the largest pituitaries were found in aged breeders. Whereas during the first year of life most adenohypophyses are symmetrical, in the second the gland tends to become nodular and haemorrhagic areas appear. Nodularity is not restricted to the very large glands weighing more than 10 mg. but can occasionally be observed in pituitaries 3·0–5 mg. In such glands one half of the anterior lobe often contains a slightly raised area which is darker than the surrounding tissue. With increasing pituitary size

TABLE III.—*Pituitary Weights of Females 6–24 Months of Age*

		Number of animals with pituitary weights of				
Age	Number of mice	–3 mg.	3–5 mg.	5–10 mg.	10–30 mg.	30–50 mg.
6–12	Virgins: 10	6	3	1	—	—
	Breeders: 18	6	9	3	—	—
12–18	Virgins: 14	3	7	4	—	—
	Breeders: 44	3	13	14	12	2
18–24	Virgins: 6	—	3	3	—	—
	Breeders: 13	1	1	4	5	2

the nodules become more numerous and the largest glands appear frankly tumorous. Such pituitaries produce signs of increased intracranial pressure. Invasion of the brain or other adjacent structures was never observed.

Histologically the largest pituitary "tumours" did not differ in any way from the smaller lesions just recognizable at autopsy except for increased vascularity and tendency for haemorrhages. Fig. 18 shows an early nodule in an asymmetrically enlarged gland and the reduction of the acidophils in one half of the anterior lobe. There is no need for a detailed description of the cytology of these pituitary growths. They resemble closely those seen in mice or rats treated with oestrogen or the spontaneous acidophilic tumours of the rat which secrete prolactin. The typical elements of these growths are cells with a large Golgi apparatus. Some contain acidophilic granules whereas others are free of them, the latter being more numerous. There is some variation in size and shape of the cells forming the nodules and mitoses can be found in moderate numbers. A section from a typical lesion is shown in Fig. 19. One additional feature is worth mentioning: the presence of foci of abnormally large acidophils with well-recognizable Golgi apparatus in areas which otherwise show the picture described above (Fig. 20). On morphological grounds one has to assume that the cells with the large Golgi apparatus and the tendency to lose their acidophilic granules, whether they occur in areas of simple hyperplasia or in adenomas, are actively secreting cells.

All animals in which a breast cancer developed in the second year of life had pituitaries weighing more than 3 mg. but in 15 females in which the pituitaries weighed from 5·1 to 51·2 mg. no breast cancer was found.

Other tumours in NZY mice

In the females, apart from breast and pituitary, the lung was the organ most frequently affected by neoplasia. All of the 20 nodules found were benign adenomas. In addition 3 granulosa cell tumours of the ovary, 2 haemangio-endotheliomas of the uterus, 2 leukaemias, 1 acanthotic papilloma of the skin and 1 β-cell adenoma of the pancreas were seen. In the 40 males 3 adenomas and 2 cancers of the lung, 2 sarcomas, 1 benign hepatoma and 1 leukaemia were found.

DISCUSSION

The enormous literature on mammary cancer in mice has recently been reviewed by Gardner (1953), Dmochowski (1953) and Lacassagne (1955). We have therefore limited ourselves to quote a few papers dealing mainly with the role of hormones in their development.

The mammary glands of NZY females become increasingly hyperplastic with advancing age and do not undergo senile involution. The rate of mammary growth is more rapid in breeders in which mammary cancers appear at an earlier age and in larger numbers. The adenohypophysis too increases progressively in size, and the largest glands have been found in mated females. The mammary and pituitary hyperplasias and the supervening neoplastic changes depend on the presence of the ovaries. That oestrogen is the ovarian factor responsible for the hyperplasia of the pituitary is most likely since in both sexes stilboestrol enlarges this gland. Proliferation of one type of functionally active acidophils seems to

account for most of the " spontaneous " enlargement of the anterior lobe. We have mentioned the similarity of these elements with the cells found in the pituitaries of rats and mice subjected to stimulation by moderate doses of oestrogen. Whereas most authors have described these lesions as chromophobic adenomas, we are of the opinion that the prevailing element is a more or less degranulated acidophil. We have classified the nodular lesions as adenomas because they were too pleomorphic to be considered as nodular hyperplasias. It is not yet known at which stage of development they become independent of ovarian stimulation. Finally we wish to stress that the pituitary lesions described in this paper are quite different from those observed by Dickie and Woolley (1949) who found the following sequence in mice gonadectomized shortly after birth : first adrenal cortical tumours appeared and later basophilic adenomas of the pituitary concurring with marked stimulation of the mammary glands.

Meites and Turner (1942) and Chamorro (1952) have shown that mammogenic potency increases in the pituitaries of rodents treated with oestrogen. Lyons and his collaborators (1953, 1955) have clearly demonstrated the importance of somatotrophin and of prolactin for mammary growth. These two hormones are secreted by acidophils. The fact that the NZY females are not larger than the males militates strongly against growth hormone being secreted by the pituitary cells which increase progressively in numbers and which form the adenomas in the anterior lobe. Mammogenic acidophilic tumours of the pituitary of the rat have been described by Lacour (1950) and by Bielschowsky (1954). Mühlbock (1953) has found spontaneous prolactin secreting tumours in mice and Furth (1955) has obtained transplantable pituitary tumours with mammogenic activity (Upton and Furth, 1955).

Prolactin has not only mammotrophic but also luteotrophic action. In most strains functionally active corpora lutea are present only during pregnancy and lactation ; but in NZY mice they must occur outside periods of gestation. Otherwise it would not have been possible to induce in young virgins deciduomas by traumatization of the uterus. Prolonged periods of dioestrus, mucification of the vagina and corpora lutea formed by large lutein cells, frequently found in females up to the age of 14 months, are evidence in favour of a relatively high progesterone secretion in NZY females.

It seems that the unusual degree of mammary development seen during the second year of life is due to the combined action of pituitary and ovarian factors. Loeb and Kirtz (1939) showed that transplanted anterior lobes of the pituitary stimulated the mammary glands and favoured the preservation of corpora lutea. The incidence of mammary cancers in virgin A mice treated with pituitary transplants rose above that of intact A breeders. More recently Mühlbock (1955) using the same procedure obtained breast tumours in virgins of 3 strains which did not possess the milk factor. In only one of these was it possible to obtain breast tumours by the administration of oestrogens. The importance of active corpora lutea in the genesis of mammary cancer in mice was clearly demonstrated by Law (1941). Symeonidis (1945) and Trentin (quoted from Gardner, 1953) demonstrated that progesterone favoured the development of spontaneous mammary cancers in this species. Recently Jull (1954) showed that the number of mammary cancers induced by a limited dose of methylcholanthrene was considerably higher in animals treated with a combination of oestrogen and progesterone than in mice injected with oestrogen only.

The breast tumours found in NZY mice show perhaps more variation in structure than is commonly seen in high mammary cancer strains carrying the milk factor. In the NZY females most of them arise in alveoli in the manner described by Bonser (1945), but neoplastic changes in the ducts are by no means rare. Often it appeared as if all epithelial elements in one field underwent neoplastic changes at the same time. Since adenoacanthomas were decisively less frequent than the small squamous metaplastic nodules discovered at autopsy it seems that only few of them progressed to the state of malignancy. Pullinger (1949, 1954, 1955) has given a detailed account of the morphology and the nature of the metaplastic lesions found in the breasts of RIII mice.

We are not yet in the possession of all the information necessary for a complete analysis of the factors instrumental in the genesis of mammary cancers in NZY females. The possible role of the milk factor is the main problem still to be solved. So far mammary cancers have failed to appear in female offspring of NZY females mated to males belonging to a low cancer strain (NZC). The hybrids have now reached an age at which breast tumours were frequent in their mothers. The presence of multiple hyperplastic nodules in the breast glands of breeders below the age of 12 months suggests that the NZY strain might carry the milk factor. However, in view of the striking particularities of NZY females in respect to pituitary and ovarian function, we are reluctant to consider these hyperplastic nodules as pathognomonic for the Bittner agent.

Of special interest were the sebaceous lesions which so far have been found exclusively in females. The susceptibility of sebaceous glands to hormones has been demonstrated by several authors. Haskin, Lasher and Rothman (1953) obtained hyperplasia of sebaceous glands in rats treated with progesterone, an effect not observed by Ebling (1947). Carlisle (1954) studied the reaction of the sebaceous glands situated in the nipple area of rabbits to daily injections of oestrone and progesterone followed by luteotrophin. Under this treatment the sebaceous glands enlarged, became multilobulated and finally gave rise to new mammary ducts. It seems therefore that in rodents an intimate relationship exists between sebaceous and mammary glands which might explain the genesis of the sebaceous lesions in abnormal mammary glands of NZY females. Chemically induced sebaceous gland tumours of the skin of mice have been observed by Twort and Twort (1930), Twort and Bottomley (1932) and recently in "Crew" mice by Rous (1956). Spontaneous sebaceous adenomas seem to be extremely rare in mice. Further investigations will be required to elucidate the role of hormonal factors in the pathogenesis of these skin tumours.

SUMMARY

An account of the origin of a new strain of mice has been given.

At present the incidence of mammary cancer in this strain is 58 per cent for breeders above the age of 6½ months and 35 per cent for virgins in the second year of life.

More than 80 per cent of intact females above the age of 6 months have enlarged pituitaries, but the pituitary weights of males and of gonadectomized animals are normal.

The mammary glands reach an unusual degree of development in virgins as well as in breeders. The excessive stimulation of the mammary gland is considered

to be due in part to an elevated secretion of ovarian hormones and in part to the mammogenic action of prolactin.

The induction of deciduomas in young virgin mice suggests secretion of progesterone by functionally active corpora lutea.

Sebaceous adenomas of the skin and sebaceous lesions in mammary cancers have been found in this strain.

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TWO CASES OF RHABDOMYOSARCOMA IN THE FOWL

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PRIMARY tumours of striated muscle are extremely rare in domesticated animals judging from the scarcity of case reports in veterinary pathology. A substantial proportion of these, seven cases in all, were found in the fowl, and it is the purpose of this paper to review these cases briefly and to describe two further examples of this interesting neoplasm.

The earliest record appears to be by Peyron and Blier (1927) who described a transplantable rhabdomyoma originating in the leg of a cockerel. This was followed by Babic (1931) who gave a brief account of a rhabdomyoma in the pectoral muscle of a young pullet. Makower (1931) cited a case recorded by Reitsma in which multiple tumours occurred in the muscles of the maxilla, thorax and abdomen of a young cockerel. Eber and Malke (1932) described multiple rhabdomyomata in the breast muscle of a hen. Meyer, cited by Feldman (1932), reported a multiple tumour arising in the pectoral muscle of a young cockerel, and finally Olson and Bullis (1942) gave an account of two such tumours in the leg muscles of an eight-month-old male and a ten-month-old pullet respectively. All these tumours occurred in the skeletal muscles of young fowls, a reversal of the situation in the human subject, where rhabdomyomata of such muscles are extremely rare. The two cases to be described conform to the previous reports of these tumours in fowls in that they also had their origins in skeletal muscle. They represent the first instance of this tumour in over 5000 spontaneous cases of neoplasia examined to date, a relative incidence of 0·04 per cent. The tumours were found in two out of a group of three young (14 week) chickens sent from the same source to a routine diagnostic laboratory for report. Material, consisting of breast muscle, ovary, segment of duodenum and pancreas, liver, spleen, kidney, bone marrow and various portions of peripheral nerves, was fixed in formal-saline and sent to the writer for microscopical examination and report. Gross examination of the tissues as received showed in the first case tumorous thickening of the duodenal wall, pancreatic involvement, multiple ovarian tumours, and several portions of muscle containing tumours. On section, one of the latter, which measured 25 × 15 × 15 mm., was seen to be a firm, greyish ovoid mass whose cut surface showed an appearance suggestive of fibroma. The second case was represented by duodenum and pancreas, liver, spleen, kidneys, marrow and portions of nerves, but only the gut and pancreas showed visible abnormality, similar in appearance to that from the first chicken. Blocks from all this material were post-fixed in susa, sections cut at 8μ, and stained with H. & E., a Picro-Mallory method, and Mallory's phosphotungstic acid haematoxylin. Glycogen stains were, of course, not possible by the time the nature of the tumour was appreciated.

Microscopic structure

The muscle tumours consisted of irregularly disposed interlacing bundles of elongated cells with eosinophilic cytoplasm and large strap-like nuclei containing finely particulate chromatin. Cross sections of the cells showed the nuclei to be mainly peripherally situated. A certain degree of cellular polymorphism was apparent, and the terminal cytoplasmic margins often had a ragged or flame-like appearance. Anastomosis between cells was occasionally noted. Although not evident in the H. & E. preparations many of these cells possessed cross striations, not always completely traversing the cell, which were plainly visible in the P.T.A.H. preparations (Fig. 1). Many of the cells also showed a fine fibrillary structure of the cytoplasm, or occasionally a lattice-like appearance. Cells cut transversely showed radially arranged striae enclosing a paler centre. Intermixed with these elements were regions of fairly dense collagenous tissue or cellular tissue with a mesenchymatous appearance. The whole tumour was richly supplied by blood capillaries. At the periphery, mature muscle formed a pseudo-capsule.

The tissue ensheathing the wall of the gut was composed of less differentiated polymorphic cells, having cytoplasmic spurs and processes, pale nuclei and prominent basophilic nucleoli. The fibrillary nature of the cytoplasm was again evident especially in transverse sections (Fig. 2). These cells lay in a matrix of rather dense connective tissue and were actively invading the pancreas and the muscular coats of the intestine (Fig. 3).

The ovarian tumour showed similar cytology, with many binucleate cells and occasional giant cells with hypertrophic nuclei. Mitotic figures were rare (Fig. 4).

In the second case, the tumour was only represented in the preparation from the duodenum and pancreas, the remaining organs being normal. However, despite the absence of a primary tumour, the histology is exactly comparable to that of the first case, with fibrillary cytoplasm, marginal hatching or fully developed radial striae in cross sections of the invading cells.

DISCUSSION

It must be assumed that the probably small primary tumour of the skeletal muscle was missed in the second case. The site of the primary in the first case is interesting, since skeletal muscle origin is extremely rare in man, the most common sites being bladder, prostate, vagina, spermatic cord, epididymis and palate, where striated muscle is normally absent (Willis, 1953). In view of this, and the almost invariable confinement of these tumours to young subjects, together with the presence in such tumours of mesenchymal tissue, Willis concludes that the usual source is not adult muscle tissue, but either embryonic myogenic cells or undifferentiated mesenchyme with aberrant striated muscle producing tendencies. This suggests that such cells are almost exclusively confined to the sites listed above in the young human subject. However, in the cases under consideration the known site of origin of one tumour was skeletal muscle, and the site in the second case was probably similar, in view of the invariably reported skeletal muscle origin in the earlier literature. It may therefore be concluded that cells with rhabdomyoblastic potencies occur occasionally in the breast or leg muscles of the young fowl. In this connection it would be interesting to compare the regenerative capacity of avian voluntary muscle after

injury, with that in mammals, where the muscle cell has lost its power of regeneration and any breach is made good with fibrous tissue. Comparative pathologists are familiar with the phenomenon of persistence of embryonic characteristics in avian tissues well beyond the first few days of life. Hepatic extra-medullary haematopoiesis, the occurrence of mixed tumours such as histiocytic and myeloblastic sarcoma, or erythroblastoma and fibro-sarcoma, the not uncommon embryonal tumours of the kidney and carcino-sarcoma of the female reproductive tract, are well known examples. Another instance, recently discovered in this Unit, is that under certain conditions, erythroleukaemia virus can initiate renal sub-capsular papillary adenomata if the chick is not older than twelve days when infected (J. G. Carr, 1956). This too, can be reasonably explained by the assumption that in the mesodermally derived kidney, embryonic elements with a wide capacity for differentiation persist for a short while after hatching.

SUMMARY AND CONCLUSIONS

Two cases of rhabdomyosarcoma in the fowl are reported, bringing the total in the literature to nine. All these cases arose in skeletal muscle. In view of the undoubted embryonic nature of the tumour it is concluded that mesenchymal tissue with myoblastic potentiality sometimes persists in the breast and leg muscles of young fowls. It is indicated that this is a further example of avian tissues retaining in immediate post-embryonic life an embryonic capacity for wider than normal differentiation, and that such undifferentiated tissue may give rise, under certain stimuli, to neoplasia.

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NOTE ADDED IN PRESS.

A further case, occurring in the cervical muscles of a pullet, and bringing the total to 10, is detailed in "Tumeurs Spontanées des Animaux de Laboratoire," p. 141 and Plate 103, by M. Guerin (1954). Amédée Legrand & Cie., Paris.

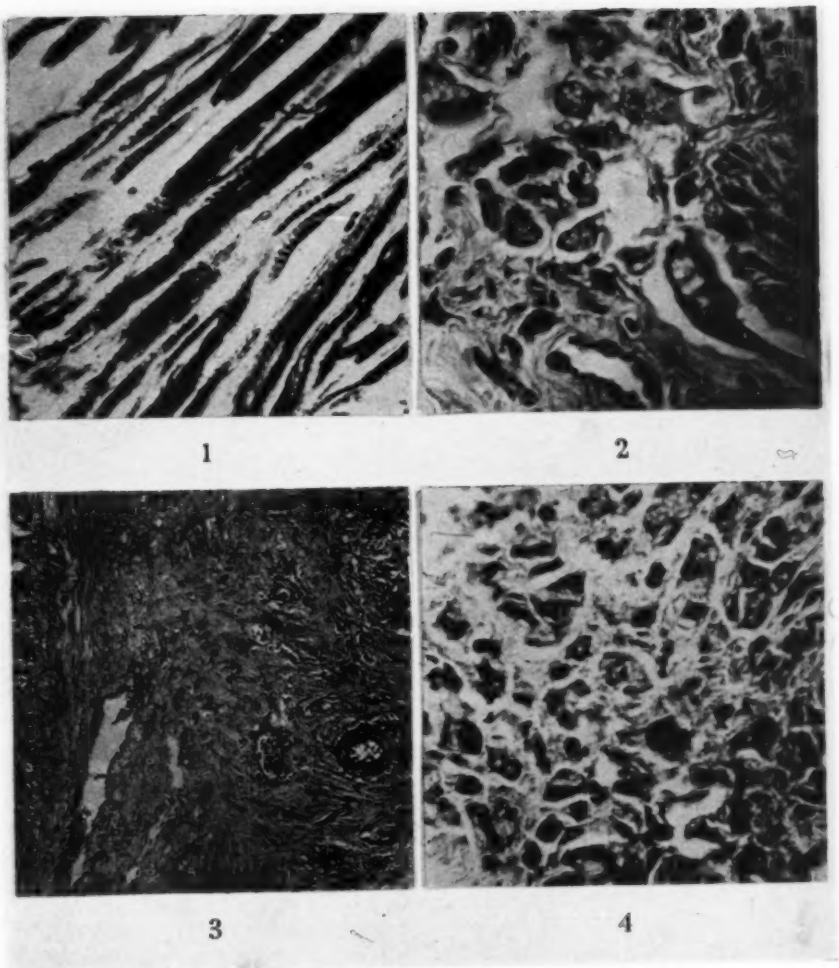
EXPLANATION OF PLATES

Fig. 1.—Tumour in breast muscle, showing cross striations in the cells. Phosphotungstic acid-haematoxylin. × 550.

Fig. 2.—Rhabdomyoblasts lying in connective tissue matrix in the tumour investing the duodenum. Note pleomorphism and the peripheral beading of the cells cut transversely. P.T.A.H. × 550.

Fig. 3.—Rhabdomyosarcoma cells invading the circular muscle coat of the duodenum. H. & E. × 60.

Fig. 4.—Structure of the ovarian tumour, showing pleomorphic and binucleate rhabdomyoblasts. H. & E. × 550.



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THE EFFECTS OF A NITROGEN MUSTARD DERIVATIVE OF PHENYLALANINE ON EXPERIMENTAL TUMOURS

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IN the search for more selective tumour inhibitors, *p*-N-di-(2-chloroethyl) aminophenylalanine, a mustatd derivative of the natural amino acid phenylalanine, was synthesised by Bergel and Stock (1953, 1954). Biological tests, using the Walker carcinoma 256 have shown this compound to be more active as a growth inhibitor in the L-form, than in the D or DL form (Haddow, 1953, personal communication). In view of this interesting finding, a study has been made of the cytological mechanism by which cessation of tumour-growth is brought about, and of the relative efficiency of the optical isomers.

Cell Injuries

(i) *Walker carcinoma*

The effects of the *p*-N-di-(2-chloroethyl)aminophenylalanine (hereafter to be referred to as PAM) has been analysed by using the transplantable Walker carcinoma of the rat. PAM was given by intraperitoneal injection of an arachis oil suspension, usually 5-6 days after implantation of the tumour graft. The tumour-bearing rats were killed at intervals varying from 8 to 120 hours. In the early experiments, PAM was used in the DL form, the dose being 2·5 mg./kg. After this dose there was a period lasting 24 hours, during which dividing cells were absent in the tumour. When mitosis reappeared, most of the dividing cells had excessive chromosome injuries which were difficult to analyse. The same dose also produced toxic effects in the cell population of the femoral marrow. For these reasons, the amount of PAM injected was reduced to 1 mg./Kg. and this dose was employed throughout except where otherwise stated.

Comparison of the cellular injuries produced by the DL, L and D forms has shown that they were the same qualitatively, and they will be described together. The duration of the mitosis-free period after a dose of 1 mg./Kg. PAM is about 16 hours in the subcutaneously growing Walker tumour and 36 hours in the same tumour growing as an ascites.

The injuries seen in the dividing cells can be grouped into 3 classes; (i) chromosome fragmentation, (ii) chromosome bridge formation and (iii) post-metaphase pycnosis of chromosomes. The last type of injury is common in tumour cells of ascitic fluid, rare in solid tumours. The chromosome fragments can be most distinctly seen in post-metaphase stages, when they lie between the two poles towards which the chromosomes move (Fig. 1, 2). Usually the "acentric" fragments, which lack the spindle fibre attachment, are not included

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in the daughter nuclei : they form the so-called " micronuclei " in the cytoplasm. The bridge configuration, seen in the ana- and telophase (Fig. 2, 3), is known to be the result of fusion of broken chromosomes in a way differing from that in the original structure. Both chromosome fragmentation and chromosome bridges are believed to lead to the degeneration of the injured cell (Koller and Casarini, 1952).

The cytological investigation has shown that the number of injured cells in mitosis and the amount of injury per cell is greater in tumour samples taken 48 or 72 hours after injection of PAM than in samples taken 24 hours after treatment (Fig. 5, 6). On the other hand, the number of dividing cells is less at 48 hours after treatment than at 24 hours. The reduction in the number of dividing cells is particularly significant in tumour samples taken 72 hours after administration of the drug. Those cells which are injured, show very excessive chromosome fragmentation, as a result of which they undergo degeneration during the post-metaphase stages and fail to complete mitosis. The process has been described as anaphase-pycnosis (Koller, 1955).

Another important change which occurs in tumours 72 hours after administration of PAM is the great increase in the number of histiocytes or stroma cells. This event is brought about partly by infiltration of these particular cells from the vascular system and partly by mitosis. Many dividing histiocytes can be seen in Walker tumours 72 and 96 hours after injection, while dividing tumour cells become very rare. In these treated tumours two other phenomena have been observed, namely the great difference in the sensitivity of tumour cells and histiocytes as shown by the amount of chromosome injuries seen in the cells and the over-development of the fibrous stroma, due to the differentiation of histiocytes.

(ii) *Other experimental tumours*

The effects of the N-mustard derivative of phenylalanine have been studied on two other rat tumours ; the " August " carcinoma and the " Yoshida " sarcoma. Both tumours were maintained by subcutaneous grafting in particular inbred strains of rats, and the Yoshida sarcoma was also used as an ascites. The August tumour was a well differentiated adenocarcinoma (Eisen, 1940) but now presents the appearance of an undifferentiated sarcoma. The growth of these tumours is slower and less haemorrhagic than that of the Walker carcinoma. We found no inhibition of growth in these tumours after the administration of PAM. Cytological analysis has shown that the number of injured tumour cells is very low indeed, being far from sufficient to bring about arrest of growth (Table I). Though the histological structure of both of these tumours differs from that of the Walker carcinoma, the failure of growth inhibition cannot be attributed to that difference alone. We observed that the tumour cells of the Yoshida ascites showed the same or nearly the same degree of response measured by chromosome injuries, as the cells of the " solid " Yoshida tumour, which was grown subcutaneously. It seems therefore, that the difference in response is due to differences which are inherent in the malignant cells of these two tumours. That this may be the case is supported by the fact that the doubling of the dose of PAM greatly increases the number of abnormally dividing cells in the Walker carcinoma, while the increase in cellular damage after doubling the dose was negligible when Yoshida sarcoma was used.

TABLE I.—*The Frequency of Injured Cells Observed After the Injection of the Mustard Derivative of Phenylalanine* (L-isomer).

(100 dividing cells are counted in each tumour).

Animal.	Tumour.	Dose (mg./kg.).	Percentage of cells injured. 24 hours.	48 hours.
Rats	Walker carcinoma (solid)	1	40	61
	Walker carcinoma (ascites)	0·25	32	51
	August carcinoma	1	7	—
	Yoshida sarcoma (solid)	1	10	—
	Yoshida sarcoma (ascites)	0·50	13	7
Mice	Krebs-2 (ascites)	2	19	12
	Ehrlich (ascites)	2	23	31
	Landschütz (solid)	2	16	12
	Landschütz (ascites)	2	30	38

The effect of PAM was also studied on two subcutaneously growing transplantable tumours of mice. Sarcoma 180 showed no response to the drug, no inhibition of growth was observed, and no chromosome injuries were seen in tumours in spite of the fact that the doses in some instances were almost lethal (5 mg./kg.). Similarly no effect was found on another subcutaneously growing tumour, MTO3, which has originated in our laboratory as a well differentiated mammary gland carcinoma and is being carried on by transplantation in strain A mice.

On the other hand it was found that the growth of three mouse ascites tumours, Ehrlich, Krebs-2 and Landschütz, can be inhibited to varying degrees. The number of dividing cells decreased 3–5 hours after administering the drugs, and the effect was quite drastic when the L isomer was used. The chromosome injuries observed in the ascites tumour cells are similar to those seen in the cells of the subcutaneously growing Walker carcinoma (Fig. 7–12). The most sensitive tumour to PAM treatment was the Landschütz ascites and it was extensively used for quantitative analysis.

The Effect of PAM Isomers on Tumour Growth

Significant differences were observed by Haddow (1953, personal communication) on the growth of Walker carcinoma between the DL, L and D forms of PAM in experiments when the drug was injected 24 hours after the implantation of tumour grafts. Similar experiments were carried out by the senior author, using both arachis oil suspensions and aqueous solutions of the sodium salts of the three isomers. The rate of growth was estimated by comparing the tumour area at different times. These data were obtained by measuring the length and width of the growing tumour and by calculating the tumour area in mm^2.

The measurements taken on the 7th, 9th and 14th day after injection show that the growth of the Walker tumour is permanently suppressed in the majority of tumour bearing animals by the L-isomer, and greatly retarded by the D-form (Fig. 13). It is interesting to note that the difference in the effects of the L and D forms became noticeable only 7 days after administration.

The frequency of injured dividing cells 24, 48 and 72 hours after the administration of the three isomers has been determined in Walker tumours, which were 5

TABLE II.—*Weight of Tumours (Walker) in Grams 13 Days after Implantation and 12 Days after 1 mg./kg. of Mustard Derivative of Phenylalanine and its Isomers.*

Arachis oil.*				Sodium salt.		
Control.	D-.	DL-.	L-.	Control.	D-.†	L-.
115	44	15	0·5	49	16	0·5
90	34	5	0	46	12	0
81	30	4	0	46	12	0
39	28	3	0	46	5	0
37	23	1	0	45	5	0
31	20	0	0	34	5	0
30	19	0	0	34	4	0
28	13	0	0	10	2	0
22	10	0	0	4	1	0
—	6	0	0	—	0	0

* Professor Haddow's test (1953).

† The growth rate of D- and L- treated tumours was the same up to the 6th day after injection—after that tumours treated with D began to grow.

days old at the time of injection. The data given in Table III show that the number of injured cells is highest in tumours treated with the L-isomer. The difference between L and D isomers was particularly significant when the drugs were administered as arachis oil suspensions. The data also reveal little difference between the efficiency of L and D isomers in producing mitotic injuries in established tumours 24 and 48 hours after treatment when the compounds are administered in the sodium salt form.

Cytological studies have also been undertaken of tumours treated 24 hours after grafting, but owing to the inflammatory reaction of the host, no reliable counts of injured cells could be made 24 and 48 hours after the administration of

EXPLANATION OF PLATES.

FIG. 1.—Late anaphase in a dividing tumour cell of Walker carcinoma showing a double chromosome fragment, which lies in the equatorial plate (24 hours after treatment with D-isomer of the mustard derivative phenylalanine : dose 1 mg./kg.).

FIG. 2.—Acentric chromosomes fragments in a dividing cell of Walker carcinoma (24 hours after treatment with the L-isomer : dose 1 mg./kg.).

FIG. 3.—Chromosome bridges at late anaphase stage of mitosis. (Walker carcinoma 24 hours after treatment with the D-isomer : dose 1 mg./kg.).

FIG. 4.—Chromosome bridges and acentric fragments in a dividing cell. (Walker carcinoma 24 hours after the DL-form : dose 1 mg./kg.).

FIGS. 5 and 6.—Excessive chromosome injuries, acentric fragments and chromosomes bridges in two dividing tumour cells ; Fig. 5 is 48 hours after ; Fig. 6 is 72 hours after treatment with the L-isomer (dose 1 mg./kg.).

FIG. 7.—Metaphase stage in a cell of Landschütz ascites showing the separation of daughter chromosomes (untreated).

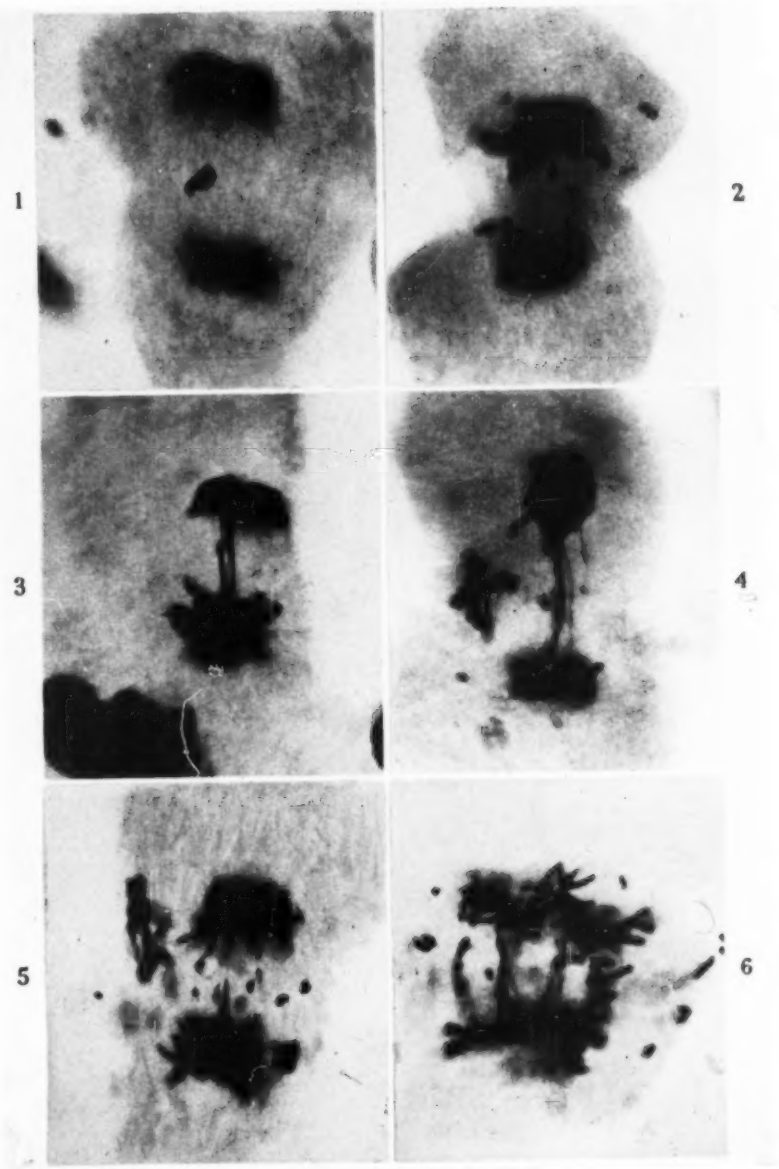
FIG. 8.—Anaphase in a Landschütz tumour cell 24 hours after treatment with the mustard derivative of phenylalanine (D-form, 4 mg./kg.). The acentric chromosome fragments fail to move towards the poles.

FIG. 9.—Late anaphase of mitosis 12 hours after treatment with L-isomer (4 mg./kg.). Besides acentric fragments, chromosome bridges are also present. The cell injury is much greater than shown in Fig. 8.

FIG. 10.—Late anaphase stage 24 hours after treatment with D-isomer (4 mg./kg.) showing chromosome acentric fragments and bridges.

FIG. 11.—Telophase of a dividing cell 120 hours after treatment with L-isomer ((4 mg./kg.) showing excessive chromosome fragmentation and the laying of acentrics on the equatorial plate.

FIG. 12.—Telophase stage 120 hours after treatment with a heavy dose of D-isomer (8 mg./kg.) showing similar type of injury as in Fig. 11.



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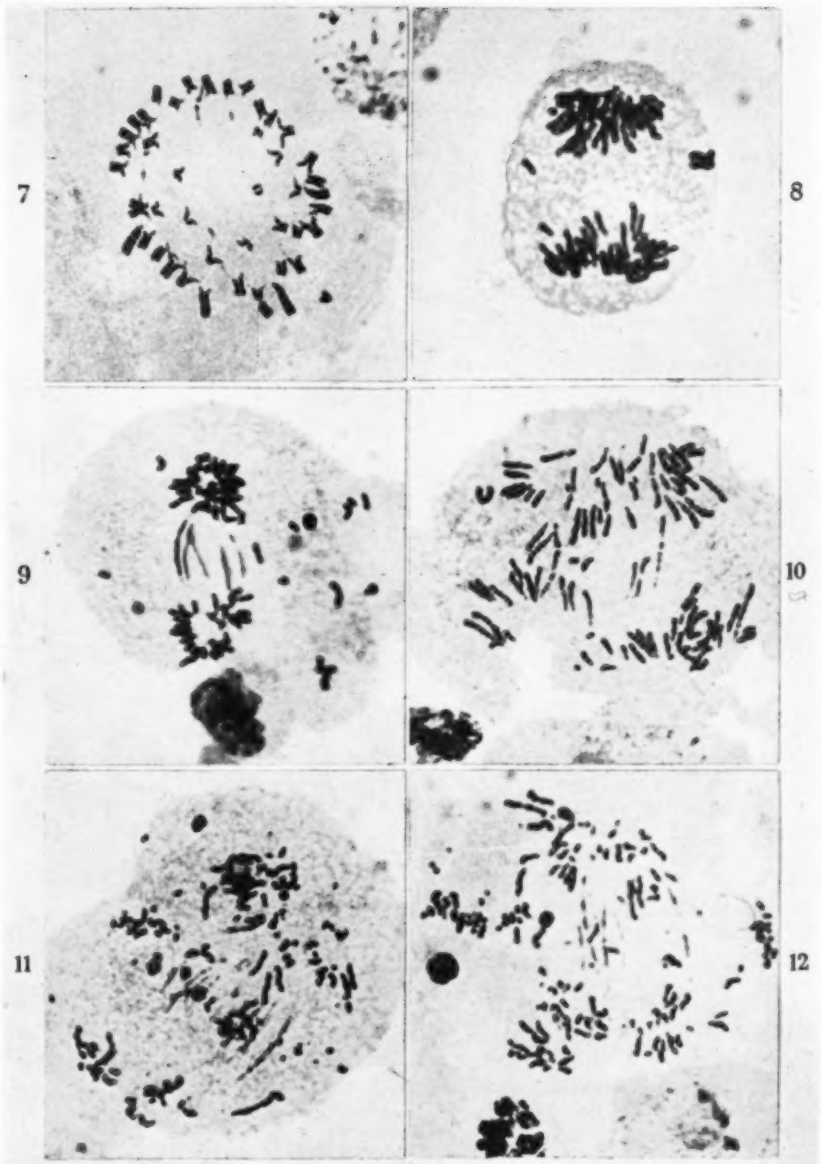

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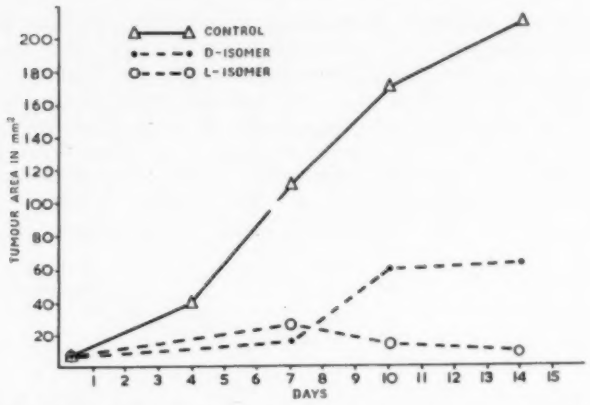

FIG. 13.—Graphs showing the rate of growth of Walker carcinoma after the injection with L- and D-isomers of the mustard derivative of phenylalanine. (Each point represents the mean of 10 measurements.)

TABLE III.—*Percentage of Injured Cells at Different Times After i.p. Injection of 1 mg./kg. Phenylalanine-Mustard Isomers.*

(100 dividing cells were analysed in each case.)

Hours after injection (in hours).	DL-isomer.		L-isomer.		D-isomer.	
	Oil.	Na-salt.	Oil.	Na-salt.	Oil.	Na-salt.
24	25	27	35	39	13	34
48	37	53	73	61	7	52
72	53	42	80	48	5	40

PAM. Data obtained in 4 days old tumours (i.e. 72 hours after treatment) showed that the L isomer is more efficient; 21 cells were abnormally dividing out of 50 counted as compared with the 13 out of 50 when the D-form was used.

The growth-inhibiting effect of the L and D isomers was also studied on the Walker carcinoma, 9 days after implantation. While no inhibition was observed in 10 tumours treated with the D-form, the growth of 4 out of 10 tumours was arrested by the L isomer.

Although the biological variables in the experiment using the Walker carcinoma are considerable (cf. Koller and Casarini, 1952), the data obtained strongly suggest a difference in the effectiveness of the L and D isomers of PAM. In order to study and to estimate the biological activity of these isomers as tumour inhibitors in more detail a quantitative analysis was made using the Landschütz ascites tumour, the growth and behaviour of which can be more easily standardised and controlled than that of the transplantable Walker carcinoma.

Cytological Behaviour and Growth Characteristics of the Landschütz Ascites.

The tumour was obtained from Dr. G. Klein of Stockholm in 1953, and was maintained by serial transfers in the inbred C+ strain. The tumour originated at the end of 1952 in Germany and was identified as a highly undifferentiated

reticulosarcoma. According to Tjio and Levan (1954) the tumour has a stemline chromosome number of 46. Our strain of Landschütz has the same characteristics as were observed by Bayreuther (1952) and Tjio and Levan (1954).

In three groups, 10 mice of the same age and weight were inoculated with 5×10^6, 20×10^6 and 40×10^6 tumour cells per ml. The growth rate of the tumour was measured by the increase in the weight of tumour bearing mice. Fig. 14 shows the weight increase after the various inocula. The difference at the three dose levels appears to be not significant. The survival time of tumour bearing mice was 17·1, 16·6 and 15·8 days for the 3 inocula respectively.

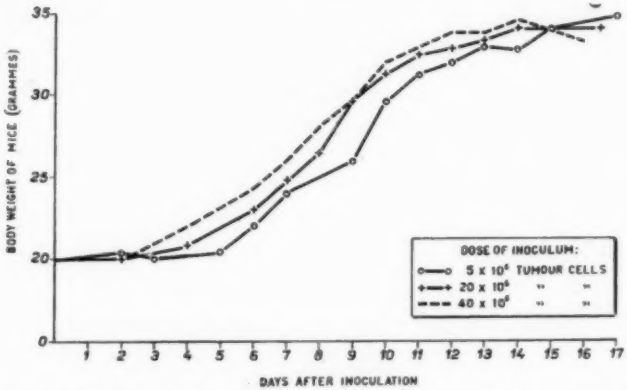
BODY WEIGHT OF MICE (GRAMMES)
35
30
25
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
DAYS AFTER INOCULATION
DOSE OF INOCULUM:
5 x 10⁶ TUMOUR CELLS
20 x 10⁶ " "
40 x 10⁶ " "

FIG. 14.—The weight of mice after the inoculation of various doses of cell suspension of Landschütz ascites.

In order to analyse more accurately the growth rate of this ascites tumour, the concentration, the total number of tumour cells, and non-tumour cells, and the mitotic index has been determined at intervals, according to the technique of Klein (1951), and of Klein and Révész (1953).

Fig. 15 shows that the number of tumour cell increases till the 270th hour after inoculation, after which the increase is very small. This coincides with a corresponding increase in the number of non-viable tumour cells : at the terminal stage the cell population of the ascitic fluid is composed of 12 per cent non-viable cells.

Effects of L, D *and* DL *forms of PAM on the Landschütz Ascites Tumours*

(*i*) *Survival time*

Five groups of mice, each inoculated with 20×10^6 tumour cells, were injected intraperitoneally with the three forms of PAM as sodium salt. The dose of the isomers and the age of the ascites at the time of treatment varied in the different experiments. The survival time of mice after the various treatments is given in Table IV. The data show that mice injected with 4 mg./Kg. L-isomer lived twice as long as the controls. The increase of survival time is less when mice are treated with the D-isomer, the efficiency of the DL-form being intermediate between that of the L and D isomers. It was also found that the same dose of the

drug is more effective when it is injected into mice soon after inoculation of the ascites cells. Thus, the development and growth of ascites could be entirely stopped in some mice, when the L-isomer (4 mg./Kg.) was administered 10 hours after inoculation of the tumour cells: in others the survival time increased to 36 days. Experiments were also performed in which PAM (L-form) was added to

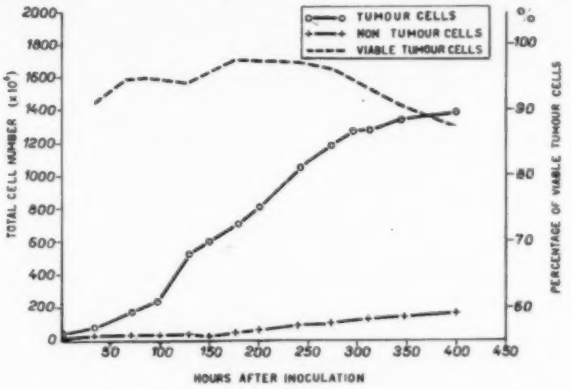

FIG. 15.—Graphs showing the total number of tumour and non-tumour cells and the percentage of viable tumour cells in Landschütz ascites (inoculum 20 × 10^6 cells). In the graphs each point represents the average of 3 measurements.

cell suspensions *in vitro*. Mice inoculated with pre-treated ascites suspensions kept alive for 9 weeks and at autopsy no signs of tumours were found.

The data of Table IV show furthermore that the effect depends also on the dose of the drug: 2 mg./Kg. was found to be less effective in prolonging life than 4 mg./Kg.

TABLE IV.—*Survival Time of Mice After Treatment with PAM*

(In each experiment 20 × 10^6 cells were inoculated.)

Dose (mg.).	Age of ascites.	Number of injections.	Survival times in days after treatment. L-form.	D-form.	DL-form.
4	10 hours	1	36·1*	—	—
4	3 days	1	30·8	25·3	27·4
4	6 days	1	27·5	—	—
2	3 days	1	21·8	19·5	—
1 (daily)	3 days	4	30·2	24·2	27·9

Control: Survival time 15·8 days.

* 5 out of 10 treated mice were without ascites after treatment.

(ii) Rate of growth

Mice were inoculated with 20 × 10^6 tumour cells and the three isomers were injected 3 days later (dose 4 mg./Kg.). The weights of mice were taken every second day and the data obtained are illustrated in Fig. 16. It can be seen that the growth of ascites was arrested by the drugs up to 12 days, and the effects of

the three forms were the same. Differences, however, became apparent after that date and the measurements showed that the L-isomer was most effective in retarding the growth of the intraperitoneal effusion.

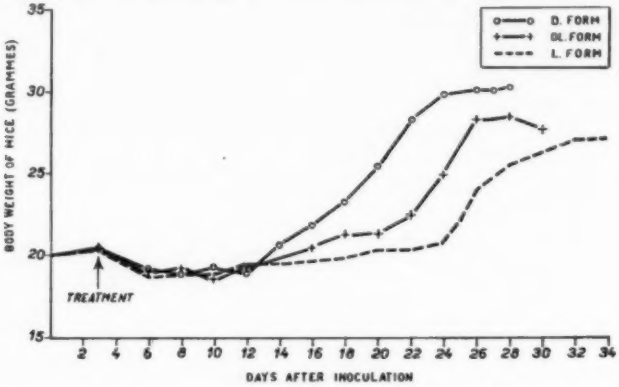

FIG. 16.—The weight of mice inoculated with 20 × 10^6 cells and treated on the 3rd day with the various isomers of the mustard derivative of phenylalanine (dose 4 mg./kg.).

(iii) *Number and viability of tumour cells*

An increase in the weight of tumour bearing mice treated with 4 mg./Kg. L-isomer of PAM was observed on the 25th day: before that day the weight remained 18–20 g. In order to analyse the cytological mechanism responsible for the delay in rate of growth after administration of the drug, the total number of tumour cells was estimated by sampling the cell composition of the treated ascites every second day. The data obtained are given in Fig. 17. The concentration and number of tumour cells remain stationary at about 20 or

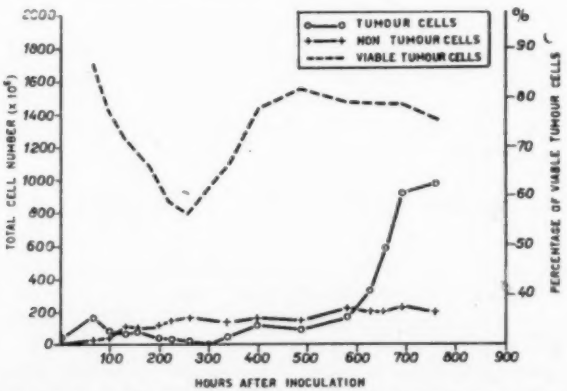

FIG. 17.—The graphs show the total number of tumour and non-tumour cells, and the percentage of viable tumour cells in the Landschütz ascites (inoculum 20 × 10^6 cells) after the intraperitoneal injection of the L-form of the drug (dose 4 mg./kg.). (Each point represents the average of 3 measurements).

30×10^6 up to 25 days (600 hours), after which there is an increase lasting 5 days. The cessation is due partly to failure of cell division of treated cells and partly to the elimination of cells which have undergone abnormal mitosis. There was a great drop in the number of viable tumour cells in ascites treated with the drug 10·5 days previously, and in the same sample 24 per cent of the cell population was found to be composed of pycnotic, degenerating cells.

(iv) *Chromosome injuries*

Mice inoculated with 20×10^6 tumour cells, were injected 3 days later with PAM. The cytological analysis has shown that the tumour cells after having been treated with the various optical forms undergo abnormal mitosis, which is due to injuries of the chromosomes (Fig. 7–12). The effectiveness of the three forms in producing abnormalities in dividing cells was measured by counting the number of abnormal post-metaphase stages. The data obtained are given in Table V. The percentages of abnormal cells after treatment with L and D isomers are compared at two doses and illustrated in Fig. 18. It seems that the two

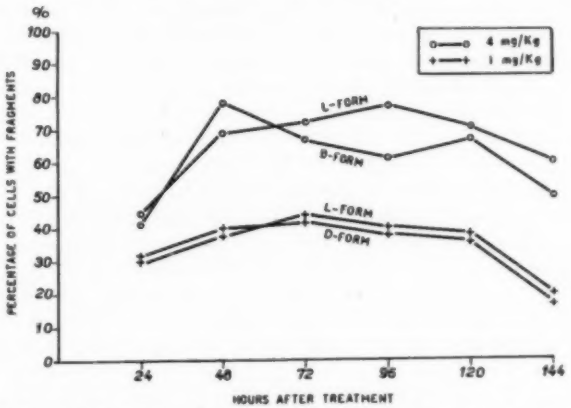

FIG. 18.—Graph showing the frequency of Landschütz tumour cells with chromosome injuries after treatment with different doses of L and D-isomers.

isomers are equally efficient in producing mitotic abnormalities when administered at a dose of 1 mg./kg. With higher doses, the L-isomer appears to produce more abnormally dividing cells than the D-isomer.

DISCUSSION

The mustard derivative of phenylalanine (PAM) has been found in our experiments to affect the growth of the Walker carcinoma when administered 24 hours after the implantation of the tumour. PAM was also tested by Larionov and his colleagues (1955) on another transplantable tumour. They used the DL form (Sarcolysine) which has been synthesised in the U.S.S.R. independently of Bergel and Stock (1953). The tumour used by Larionov was sarcoma 45 which was produced by 9, 10-dimethyl-1,2-benzanthracene. Sarcoma 45 kills the tumour-

bearing animals on the 25–30th day after implantation : i.e. its rate of growth is slower than that of the Walker carcinoma. Larionov reported that a single intraperitoneal injection of 15 mg./kg. of the drug was sufficient to suppress the growth of this particular tumour completely. As a result of treatment the histological structure of sarcoma 45 undergoes a drastic change : the small residual nodule left at the site of growth, was found to be composed merely of connective tissue stroma devoid of tumour cells.

The L-isomer has also been tested against the Harding-Passey melanoma grown in mice of *dba line*-1. The growth of 9 days old melanoma was arrested by an intraperitoneal injection of the L-form of PAM (dose 14 mg./kg.) (Luck, 1956). In the present investigation complete regression as well as temporary inhibition of growth has been obtained with PAM in the Walker, Ehrlich, Krebs and Landschütz ascites tumours. The suppression of growth was always found to be preceded by cell injuries, very similar to those seen in the Walker carcinoma after HN2 treatment (Koller and Casarini, 1952).

Comparison of the various effects which appear after the administration of the three isomers of PAM indicate that the L-form is the most effective, PAM in this form stops the development of Walker carcinoma at least in 80 per cent of the tumour-bearing rats. Similar observations were made when the effects of the various isomers on the growth rate of the Landschütz tumour and on the survival time of tumour-bearing mice were compared : in both respects the action of the L isomer was greater than that of the D or DL forms.

On the other hand, when tumour cells with chromosome injuries are used as the criteria of activity, the difference between the various isomers appear to be too small to account for the significant differences in growth rate. The number of injured cells in tumours treated with L, D, and DL forms appears to be similar except when the drugs are administered as arachis oil suspensions, in which case the superiority of the L isomer becomes more obvious. It seems therefore that growth inhibition is not due to the death of tumour cells only which is brought about by chromosome injuries : other events, e.g. suppression of mitosis, cell pycnosis in resting stage, stromal reaction etc. which follow the administration of the drugs also play a role in growth suppression. It has been observed that these processes differ in expression, intensity, time of onset and duration in a 6–7 days old rapidly growing Walker carcinoma treated with the various isomers of PAM. At present, however, no method is available to compare these phenomena on a quantitative basis.

Furthermore, it has been found that 24 or 48 hours after implantation the migrating and proliferating cells of the Walker carcinoma graft are under the influence of an inflammatory local reaction and that during this process the effects of drugs (e.g. HN2) differ in type and intensity from those effects which the same drugs produce in a 6–7 days old well-established tumour (Koller, unpublished). Some evidence has been obtained which seems to indicate that in a young tumour graft the onset of mitosis suppression occurs 6 hours later after the administration of the D isomer than after that of the L-form of PAM. Such a delay during the crucial period of tumour establishment (" take ") would alone be sufficient to account for the difference in the rate of growth at later stages of tumour development.

The mean lethal dose (LD_{50}) in male rats was found to be about 10 mg./kg. for the L-form of PAM while it was 30 mg./kg. for the D-isomer (Boyland and

Seeking, personal communication). The difference made it possible to increase the dose of D-isomer in some experiments with the aim of producing the same number of cells with chromosome injuries, and the same degree of growth inhibition in the Walker carcinoma as was found after the administration of the L-isomer. The data obtained suggests that 3 mg./kg. of D is equivalent to 1 mg./kg. of L in producing the same number of cells with chromosome injuries in the Walker tumour 24 and 48 hours after intraperitoneal injection. In order, however, to suppress the growth of a 24 hour tumour graft, it was necessary to increase the dose of D isomer to 5 times that of L-form.

Thus the various biological tests, when they are considered together, strongly suggest that the L-form of PAM is more active as a growth-inhibitor of some particular transplantable tumours than either the D or DL forms. The question naturally arises as to what are the differences between the isomers which could account for the differences in biological activity. If for instance there were a difference in the rate of transportation, excretion, etc. between the L and D forms, they might be absorbed differentially by the tumour cells. In the present investigation some differences have been noted which would suggest that the physical properties of the isomers may play a role. It was mentioned previously that the difference in the number of injured cells was greater when the two isomers were administered in arachis oil suspensions and not in the sodium salt form (Table III). Similarly at high dose levels the difference between the L and D forms in producing cell injuries in the Landschütz ascites tumour, was greater than at low dose level (Table V and Fig. 18).

TABLE V.—*Percentage of Anaphases with Fragments in Landschütz Ascites Treated with the Isomers of PAM.*

(100 dividing cells were counted in each cell).

Dose (mg./kg.).	Isomeric form.	Per cent of cells with fragments.					
		24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	144 hr.
8	L	64	85	81	76	—	—
	D	55	71	76	78	—	—
4	L	45	69	72	76	71	60
	D	42	79	67	61	66	51
	DL	40	73	—	—	46	65
2	L	42	—	—	—	—	—
1	L	30	38	44	40	38	20
	D	31	40	42	37	35	18
0·4	L	9	19	4	9	6	6

These few facts seem to suggest that the mustard derivative of phenylalanine may act in cell metabolism as the L-isomer : i.e. the D form must be racemised before it can interact with cellular enzymes or intermediates. It is, however, possible that the D-form of PAM is active as such, but owing to its stereochemical configuration the incorporation of this isomer into cellular metabolites is slow. Both processes would introduce a time lag : some loss would occur if racemisation were involved since this, would result in the lower biological activity of the D isomer observed in our experiments. The differences seen in the time of onset of

mitosis suppression in a 1 day old Walker tumour graft after the administration of L and D isomers suggests that this may be the case.

SUMMARY

1. A nitrogen mustard derivative of phenylalanine inhibits the growth of certain experimental tumours in rats and mice.

2. The growth inhibition is initiated by injuries to the chromosome mechanism, which leads to the death of tumour cells.

3. Cells in malignant effusions are more sensitive to the drug than cells in the subcutaneous growing solid tumour.

4. The L-isomer is more effective as an inhibitor of the growth of the Walker carcinoma than the D or DL forms.

5. In producing chromosome injuries in the Walker tumour cells the L-isomer is 3 times more active than the D form and it is 5 times more effective as a growth inhibitor of this tumour than the D-isomer.

6. The difference in activity between the L and D or DL forms is greater when the drug is administered in arachis oil suspension.

7. The drug increases the survival time of mice inoculated with the Landschütz ascites tumours. The L-isomer was the most effective, prolonging life to 36·5 days as compared with 15·5 days for control mice. The D and DL-forms were less effective in this respect than the L-form.

8. In both the Walker carcinoma and the Landschütz ascites, the differences shown by the three isomers on growth inhibition becomes measurable 7 and 12 days respectively after administration of the drug.

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ATTEMPTS AT ORAL TRANSMISSION OF MAMMARY TUMOUR AGENT BY FEEDING MALE ORGANS

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THE male mouse harbours the mammary tumour virus in its testes, spermatozoa vesicular glands and fluid (Andervont and Dunn, 1948 ; Mühlbock, 1950) as well as in other sites. Male secretions therefore might be a source of infection to female offspring either by direct contact or by infection of their mothers.

Mühlbock (1952*a*) suggests that repeated copulation by increasing the mammary agent transmitted by a " high cancer " male to a " low cancer " female eventually leads to a high enough concentration of agent for the mother to transmit it to her young in the milk. He also injected extracts of embryos removed from high cancer strain mothers with mammary tumours into 3 to 4 weeks old susceptible mice (Mühlbock, 1952*b*). These mice did not develop any mammary tumours, in spite of being force-bred ; while 50 per cent of mice injected with placental extracts from a similar source developed mammary tumours. Though these experiments do not exclude absolutely the contamination of the young through the placenta, they support the concept of indirect infection by the mothers' milk. The possibility remains that unexplained mammary tumours might be due to chance oral contamination of the offspring in the cage when coming into contact with some secretion of its male parent

In the human subject the mumps virus localises in the mammary gland, parotid and testes, and this association suggested that a similar tropism for these glands may exist in the case of the mammary tumour agent in mice. In the following experiments it was proposed to test the possibility of contamination by salivary, testicular or vesicular gland secretions.

Feeding methods

Vesicular gland.—The males were killed by cervical dislocation, the vesicular gland was rapidly exposed and a fine glass rod was dipped repeatedly into its lumen to collect the secretion which was then inserted into the mouths of the young mice. In some cases scrapings from the wall were included in the feeding. The feeding was not done quantitatively.

Testes and salivary glands.—Small fragments of glands, as well as some tissue fluids and secretion, were introduced into the mouth. Blood was avoided as far as possible.

Experiment I

Twenty-four RIIIf (previously referred to as RIIIb) mice and their litter mate brothers were fed between 6 and 120 hours after birth with vesicular fluid, and four with testis, of C_3H mice. These C_3H males (about 12 months old) were brothers or sons of mammary-tumour-bearing mice.

After being fed, the offspring were returned to their parents until weaning time, after which each litter was left to force-breed between brothers and sisters until up to 12 litters were produced. Males and females were left continuously in the cage until death, and their litters were killed shortly after birth, except when they were killed and eaten by their own parents.

Experiment II

(*a*) Six C57 female mice about 48 hours old were fed with fresh testes from C_3H males. (*b*) Six C57 female mice of the same age were fed with fresh salivary gland from the same males used in (*a*). Both groups were force-bred as in Experiment I above.

After 19 months all the mice are alive and none shows any tumour, nor do their C57 mothers, now 26 months old.

TABLE I.—*Fate of RIIIb Female Mice Fed with Vesicular Fluid and Testis from C_3H Males*

Mouse No.	Age when fed (hours).	Age at death (days).	Total number of litters.
1	6	606	9
5	12	630	8
6	12	630	5
18	12	693	3
19	12	670	6
20	12	693	6
11	24	371	8
21	24	717	6
22	24	796	7
25	24	714	7
2	48	625	3
3	48	515	2
4	48	138	2
*14	48	520	12
*15	48	418 (MT)	9
*16	48	641	5
*17	48	622	4
26	72	552 (MT)	6
27	72	823	6
28	72	826	8
24	72	564	6
7	96	784	4
8	96	564	6
9	96	776	3
10	96	336	4
12	96	663	10
13	96	461	9
23	120	687	10

* Testis fed.
MT = Mammary tumour.

RESULTS

In the first experiment 2 out of 28 female mice developed a mammary tumour. One (fed with testis) was killed at 418 days bearing an epidermoid carcinoma on the right side at the level of the 4th breast, while the other (fed with vesicular fluid) was killed at 552 days with a fibroadenoma of the right side at the level of the 3rd breast.

DISCUSSION

Previous experiments with susceptible females borne by supposedly virus-free mothers from virus-carrying fathers suggested that the presence of the father at the time of birth and subsequently might in some way provide a source of infection leading to the development of a mammary tumour in the offspring (Peacock, 1953). But it was shown by Pullinger (1953) that some CBA females reputed to be virus-free were sporadic carriers of the agent. In the present experiment neither parent carried the virus and the only possible source of virus was the vesicular fluid or testes of the high mammary cancer strain used for feeding.

The tumour incidence, 2 out of 28, does not exceed the expected amount seen in RIIIf mice which have lived to a similar age and borne over 5 litters (Pullinger, 1955). Moreover the histology of the tumour, an epidermoid carcinoma and a fibroadenoma with sarcomatous changes in the stroma, differs from the adeno-carcinomatous type characteristic of virus-induced mammary cancer in mice.

Vesicular gland sections stained by the phloxine tartrazine method failed to reveal inclusion bodies, but extensive examination of mammary glands and tumours from high cancer strain did not show either any characteristic structure of this kind (Pullinger, 1956, personal communication), though the mammary gland in the high cancer strain mice is an accepted source of mammary tumour agent.

There is a physiological similarity in the method by which mammary gland and vesicular glands discharge their products.

Biancifiorni, Lotti and Squartini (1954) have described an apocrine secretion of the cellular layer in the male reproductive glands which would allow the discharge of the agent and of normal macromolecules with the shed cells into the lumen of the gland. However the vesicular fluid or testes differ from semen and selection of only one of the components, as in these experiments, might fail to provide an active virus.

In all such experiments the problem of what constitutes an infective dose has to be considered. It has been shown that about 0·1 ml. of milk from a high mammary cancer strain is sufficient to infect susceptible female mice up to 48 hours of age, of high cancer strain (C_3H) fostered on C57 (Andervont, Shimkin and Bryan, 1942), or low cancer strain C57 fed with RIII milk (Haagensen and Randall, 1945).

Graff *et al.* (1949) report carcinoma of the breast in mice injected intraperitoneally with particulate fractions obtained by differential centrifugation of high cancer strain milk containing as little as 8 mγ of nitrogen. But such quantitative data refer to material of undetermined viral content and Huseby, Barnum and Bittner (1950) noted great variations of infectivity in lactating mammary glands of different mice of the same inbred strain.

In the natural transmission of the mammary tumour virus from mother to offspring the milk provides not only a source of virus but also of oestrogens and possibly other hormones which favour infection. Presumably such hormonal factors are still provided by the milk of the virus-free females of high cancer strain. It was thought therefore that offspring of such RIIIf mothers would provide the ideal subjects for the reception of virus from the C_3H male source.

The negative result of this experiment could be explained by the absence of a virus or its presence in subinfective amount.

SUMMARY

Oral feeding of vesicular fluid or testes of C_3H mice to RIIIf females did not cause an increase of mammary tumour incidence in excess of what is expected from an average breeding female of this strain.

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EFFECT OF VARIOUS CHEMOTHERAPEUTIC AGENTS ON METASTASIS

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EXPERIENCE shows that in infective and tumoral diseases (Druckrey, 1955; Yoshida and Ishidate, 1955) resorptive chemotherapy fails to bring about definite recovery unless the pathogens or the tumour cells (which as regards the production of recurrence and metastases are also pathogens) are scattered in clumps over the organism or circulating freely in the blood stream. Localised processes, among them large-sized solid tumours, primarily require local intervention, while chemotherapy should strive in the first instance to combat the minute agglomerations of cancer cells dispersed in the organism.

Accordingly, instead of the inhibition by chemotherapeutic drugs of growth in solid tumours, in the present work the effect of these agents on the production of recurrence and metastases is studied.

With a view to producing an experimental model, animal organisms were flooded with cells from a florid Guérin T108 carcinoma of the rat, known to be highly resistant to chemotherapeutic drugs.

To begin with, the effect which 1, 6-bis (β-chloroethylamino-1, 6-deoxy-D-mannite)-dihydrochloride (BCM) exerts on the production of metastases was studied, because this nitrogen-mustard derivative seemed to be equally promising on the evidence of animal experiments (Kellner and Németh, 1956) and clinical observations (Sellei *et al.*, 1956). Favourable results having been obtained with it, the investigations were extended to other radiomimetic substances which experimental and clinical data likewise showed to be possibly efficaceous. These included: HN_2N-oxide (di-2-chloroethyl methylamine oxide; Nitromin), triethylene-melamine (TEM), 1 : 4-dimethanesulphonyloxybutane (Myleran), and a few other chemotherapeutic drugs of a different nature such as deacetylmethyl-colchicine (Colcemid), Teropterin, and stilboestrol-diphosphate (Honvan). Cortisone (11-dihydro-17 hydroxycorticosterone-21 acetate), which is known to intensify metastasis production, was used in combination with some of the other drugs to enforce their maximum performance.

MATERIALS AND METHODS

Male and female rats of the Debrecen strain weighing 150 to 200 g. were each inoculated intravenously with 3 million cells of Guérin carcinoma, and divided into groups of ten. Shortly after the inoculation the animals received the first injection of the drug to be tested, in doses considered to be suitable according to the data in the literature and our own preliminary experiments. Dependent on their condition, the animals were given 6 to 20 injections. The drugs were administered intravenously, except those only soluble in oil, which were introduced

TABLE I.

Group (Number of animals in parentheses)	Dose.	Average of changes in weight (g.)	Average survival time (days)	Metastatic conditions								Number of animals with tumours	Notes
				Lungs	Liver	Kidneys	Adrenals	Sexual glands	Thoracic lymph nodes	Abdominal lymph nodes	Body-surface lymph nodes		
*Control (40)	—	−25·1	28·7	23	—	13	9	6	35	33	18	40	—
Teropterin (10)	15×2 m./kg. i.v.	−7·6	29	4	—	3	2	—	9	9	5	9	—
Honvan (10)	15×50 mg./kg. i.v.	−39·2	27·1	2	—	5	4	2	9	7	5	10	—
Myleran (10)	5–8×5 mg./kg. i.p.	−43·1	17·1	2	1	1	—	—	2	1	2	5	3 animals died early, within first 15 days.
Cortisone (10)	15–30 ×2 mg./kg. subc.	−59·9	28·1	9	7	3	7	3	9	7	2	9	1 animal sacrificed early.
Colcemid (6)	12×1 mg./kg. i.v.	−37	42	5	2	2	3	—	5	5	3	5	—
Colcemid (6)	10×2 mg./kg. i.v.	−62	28	6	4	3	2	—	4	4	4	6	—
Colc.+cort. (6)	—	−62	28	6	4	3	2	—	4	4	4	6	—
TEM (10)	14×6·2 mg./kg. i.p.	+ 7	41·7	3	—	1	1	1	8	6	6	9	1 animal died early.
Nitromin (10)	6×20 mg./kg. i.v.	−41	22·4	—	—	—	—	—	—	3	3	3	4 animals died early, within first 12 days.
Nitromin (6)	15–20 ×10 mg./kg. i.v.	−19	40	1	—	2	2	—	3	2	—	4	—
*BCM (28)	15–30×10–15 mg./kg. i.v.	−32·6	44·2	—	—	—	—	—	—	—	—	—	—
BCM (10)	6×20 mg./kg. i.v.	−44	18·3	—	—	—	—	—	—	—	—	—	5 animals died early, within first 15 days.
BMC+cort. (10)	15–20×2 mg./kg. cort. subc.+10–15 mg./kg. BCM i.v.	−34·6	25·1	—	—	—	—	—	—	—	—	—	

* Combined data of several groups.

intraperitoneally. Death of the animals was as a rule awaited. The macroscopic findings concerning generalisation of the tumour were in each case controlled histologically.

RESULTS

Tumours developed without exception in all the untreated control animals inoculated intravenously with Guérin T108 rat carcinoma. In 2 to 3 weeks after the inoculation, extensive metastases were found to have been produced in many different internal organs and practically all the lymph nodes, and the animals died with considerable loss of weight within 30 days. In no case was spontaneous regression encountered. From Table I it can be seen that Teropterin and Honvan exerted no influence whatsoever upon the development of the tumour, the formation and distribution of metastases, the survival or the body weight of the animals. The situation was essentially the same with Myleran, but it should be mentioned that the proper evaluation of this drug was rendered difficult by the early death of the animals due to its toxicity. As was to be expected on the ground of other workers' findings (Agosin *et al.*, 1952; Molomut *et al.*, 1952; Pomeroy, 1954) and our own earlier experience (Lapis and Sagi, 1956) cortisone intensified tumour generalisation and metastases arose in organs (liver) where none was ever encountered in the control animals. In addition, it caused the animals to deteriorate in condition to an even greater extent. Colcemid in 1 mg. doses was found to prolong the survival time, but to be unable to inhibit tumour generalisation or extensive metastasis either in 1 or in 2 mg. doses. On the contrary, it had an adverse effect, for, like cortisone, it frequently gave rise to metastasis in the liver and deteriorated the condition of the animals. Combined treatment with cortisone and Colcemid yielded essentially similar results.

More favourable results were achieved by treatment with TEM, Nitromin, and BCM. It was found that TEM prolonged the survival time, yet ultimately all animals treated with it died from generalised metastases. Tumour generalisation was considerably less pronounced after administration of Nitromin; no metastases developed in the internal organs, and although the average survival time was substantiallly longer, some animals, even died entirely free from tumours. In our previous experiments it had been found that BCM significantly reduced the incidence of metastases from subcutaneously inoculated Guérin cancer; the present experiments showed that it checked tumour progression under different conditions as well. It inhibited generalisation and extensive metastasis production after intravenous inoculation of the tumour, and in some cases even led to complete recovery. Combined with cortisone it warded off the latter's tendency to intensify the development of metastases. In appropriate dosage it prolonged survival time.

DISCUSSION

Insufficient attention has been devoted to the experimental study of the effect of chemotherapeutic drugs on the production of metastases, probably because of the absence of an adequate experimental model closely mimicking the metastatic conditions usual in human pathology. In the present experiments subcutaneously inoculated Guérin cancer served as the model for lymphogenic metastasis production (Lapis and Németh, 1956*a*, 1956*b*), which systematically led to the development of extensive lymph-node metastases. The haematogenous

metastatic condition resulted from the intravenous inoculation of the Guérin carcinoma.

This time, we experimented solely with drugs of known activity, on the evidence of experimental or clinical data. Of the agents studied, only certain radiomimetic substances yielded favourable results (BCM, Nitromin, TEM), especially BCM, which apparently surpasses all other known nitrogen-mustard derivatives owing to its slight toxicity, wide therapeutic range, prolonged action, and increased tumour affinity (Kellner, Németh and Sellei, 1956). Myleran, though also radiomimetic, appeared ineffective, but it should again be noted that because of the early deaths due to its great toxicity, the drug was difficult to evaluate. The fact that its chemical structure differs from that of the halogenethyl derivatives may be responsible for its inefficacy. For the present we are unable to offer an explanation for the complete inefficacy of Teropterin, but the ineffectiveness of Honvan was not surprising since it is known to have a rather peculiar mechanism of action and, in a certain sense, a selective effect. On the other hand, the unfavourable effect displayed by Colcemid was very unexpected, the drug being the detoxicated derivative of colchicine which is one of the most powerful mitotic poisons known to-day (Santawy and Reichstein, 1950). Although experiments with mice have shown the toxicity of Colcemid to be many times less than that of colchicine, it possesses the same power quantitatively and qualitatively to inhibit cell division in tissue cultures and the growth of certain transplanted tumours; including according to data in the literature, Guérin T108 rat carcinoma (Schär, Loustalot and Gross, 1954). The observation that the effect of Colcemid on Guérin cancer is strong if the tumour is inoculated subcutaneously, but absent, or even adverse, if the inoculation is intravenous, points to biological differences in behaviour on inoculation by different routes, and seems to afford support for our contention that from the point of view of metastasis inhibition the biological alkylating agents are those most worthy of consideration.

The favourable results obtained with some of these drugs in model experiments lead to the hope that they may be turned to good account in clinical practice where conditions similar to those in the model experiments usually arise after surgical removal of the tumour, i.e., when the presence in the organism of a sizeable solid tumour need no longer be feared, but transient carcinomatous cytaemia (Fischer, 1955) and remaining minute cancer cell clumps disseminated over the organism and inaccessible to the surgeon (Kraus, 1954), become of principal concern. It would appear that with the aid of some of the drugs studied in our model experiments, especially with BCM, it might become possible to combat successfully these disseminated tumour-cell colonies; and if surgical intervention is carried out under the protection of these chemotherapeutic agents, to forestall later recurrence or metastases, which are the greatest obstacles to the final success of operations for cancer.

Most naturally, animal experiments in no way decide which of these drugs may be the soundest to use in clinical practice, but they can serve as a guide to clinicians on this point, as well as in suggesting the most expedient mode of application.

SUMMARY

1. The effect of various chemotherapeutic drugs (BCM, Nitromin, TEM, Myleran, Colcemid, Teropterin, Honvan, cortisone) upon the production of metastases in animals has been studied.

2. It has been found that Teropterin, Honvan, and Myleran, do not essentially affect generalisation of intravenously-inoculated Guérin T108 rat carcinoma, or the survival time, and that Colcemid, like cortisone, exerts effects that are rather adverse than favourable.

3. Only the radiomimetic substances BCM, Nitromin, and TEM, have been observed to produce favourable effects; principally BCM, which inhibited generalisation of intravenously-inoculated tumours and the establishment of multiple metastases; moreover, when combined with cortisone in the treatment, it warded off the latter's tendency to intensify metastasis production; it also prolonged the survival time of the animals.

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THE OCCURRENCE OF METASTASES IN A GROUP OF RELATED RAT TUMOURS

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MANY experimental tumours that are classified histologically as malignant have never been observed to metastasize. For example, examination of an extensive survey of transplantable tumours published by Dunham and Stewart (1953) shows that only about forty per cent of the carcinomata and sarcomata listed have been reported to metastasize at all. This fact has often been attributed to the rapid local growth which causes the death of the host before metastases have a chance to develop. From time to time, however, the validity of the diagnosis of malignancy of some transplantable tumours on histological grounds is challenged in view of their behaviour, and doubt is expressed as to what extent they may be compared with human malignant neoplasms, which tend to consist of a relatively small primary growth when compared with animal tumours, and which usually spread extensively before death ensues.

One group of animal tumours which may show the histological appearance of malignancy, but from which no metastases have been reported are the sarcomata that arise in transplantable fibroadenomata in rats. These have been described by several workers including Loeb and Fleisher (1916), Heiman (1934), Emge (1938), Selbie (1941), and most recently by Millar and Noble (1954*a*). All of these investigators have reported the development of sarcomata in benign transplantable fibroadenomata which arise in the breast tissue of female rats. These sarcomata vary considerably in their appearance and behavior, but in general are fibrosarcomata with a fairly rapid rate of growth and are transplantable to males and females. Invasion of the surrounding tissues may occur, but no metastases have been reported by any worker, although Selbie (1942) described a single instance of a carcinoma arising from a fibroadenoma and producing distant metastases. It seems unlikely that all of the workers quoted carried out an exhaustive search for metastases : nonetheless, pulmonary nodules are not as a rule difficult to identify, and the lungs represent the commonest site for sarcomatous metastases. It is felt that the clear statement by the foregoing writers that metastases do not occur cannot be disregarded merely because proof of a very detailed study of all organs is lacking.

Since a variety of these sarcomata, all of which arose in the same transplantable fibroadenoma, were available for study in this laboratory, it seemed of interest to determine whether any of the tumours, particularly those which were histologically very malignant in type, would metastasize under any circumstances. Studies on the subject of experimental tumour spread remain somewhat meagre,

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and it was felt that such an investigation might indicate what factors were most critical in its occurrence.

Factors determining whether metastases occur might be classified as follows:

1. Intrinsic properties of tumour cells which one may assume to be present in some tumours and absent in others. These include the ability to invade vascular channels, to survive transport through the blood stream, and to establish a new focus of growth upon lodging again in smaller vessels. It is possible that the possession of these properties may vary, not only among different tumours, but among different cells of the same tumour. Growth rate of the neoplasm may be an additional important intrinsic factor, although the entire picture as concerns the effect on metastases of growth rate, life span and final tumour size is not clear. Zeidman, McCutcheon and Coman (1950) reported that the number of metastases was directly proportional to the duration of primary tumour growth, and bore no relationship to final tumour size. This suggests that a slow growth rate would favour metastasis. Wood and his co-workers (1954) however, showed that with a constant inoculum over a given time interval, the number of metastases was directly proportional to the final tumour size. Similarly, the work of Martinez, Miroff and Bittner (1956) indicates that the incidence of metastases was highest in rapidly growing tumours, in spite of the fact that they remained in the host for a shorter period of time. In initiating the present study it was hoped that differences might exist among the tumour sublines as to growth rate, ability to invade vessels, and ability to produce tumours when injected intravenously, which might suggest which fundamental properties were lacking in these tumours. At the same time, the sublines have a common origin in a single neoplasm and should be therefore directly comparable in a transplantation study.

2. Varying environmental conditions under which the same neoplasm is growing. These include the general condition of the host as regards nutrition, hormonal balance, the composition of the blood, irradiation effects, and systemic defences of the host (if any exist apart from those directed against genetically incompatible tissue). The effects of some of these factors have been studied extensively. Cortisone has been reported by several groups to increase metastatic frequency (Agosin *et al.*, 1952; Baserga and Shubik, 1954; Pomeroy, 1954; Wood, Holyoke and Yardley, 1956) while others have disputed this claim (Kaliss, Borges and Day, 1954: Goldie, Walker, Jeffries and Guy, 1955; Martinez and Bittner, 1955). Wood, Holyoke, Sommers and Warren (1955) have described a stimulating effect on metastases from growth hormone. Tannenbaum and Silverstone (1952) reported a reduction in metastases when the food intake of the host is limited. Anticoagulant therapy has been reported to decrease the number of metastases from intravenous tumour inoculation (Terranova and Chiossone, 1952; Wood, Holyoke and Yardley, 1956). Blood loss as a factor in promoting metastases has been cited (Gaylord and Simpson, 1916; Tadenuma, 1923; Tadenuma and Okonogi, 1924); although the evidence cited by Gaylord and Simpson is somewhat meagre.

Whole body irradiation has been reported to result in increased metastases (Cirio and Balestra, 1930) although other workers (Von Essen and Kaplan, 1952) have found that while irradiation of the tumour increases metastases, irradiation of the host alone was without effect. The effect of sex hormones on tumour growth has, of course, been very widely studied; their effect on metastasis

formation seems to have largely escaped attention. The type of tumour used in the present study has shown comparatively little effect from hormone alterations in the host (Millar and Noble, 1954*b*). Studies on the effect on metastases of general systemic changes such as the foregoing were not included in the present investigation.

Other variable factors affecting the tumour environment are more local in nature and include the influence of the site of the transplantation, and the effects of mechanical interference such as incision, subtotal removal and massage. Intraperitoneal grafts are said to produce more frequent metastases than intramuscular or subcutaneous transplants (Mazzacuva, 1933 ; Goldie, Jeffries, Jones and Walker, 1953), as are grafts into the tail (Bonne, 1925 ; Baserga and Baum, 1955). Subtotal removal of a tumour likewise, has been reported to increase metastases (Clunet, 1910 ; Roussy, Oberling and Guerin, 1936 ; Crabb, 1949). Massage of a tumour has been shown to augment metastases (Tyzzer, 1913 ; Knox, 1921 ; Marsh, 1927). It may be noted that such procedures have been carried out almost exclusively on tumours capable of occasional spontaneous metastases under normal circumstances : no demonstration of metastases by these means in a tumour normally unable to metastasize has been found. The situation when intravenous injection of such a tumour is made is, however, not entirely clear. The works of Weil (1913) and of Andervont and Shimkin (1941) are strong evidence that tumours incapable of spontaneous metastases can be made to produce pulmonary tumours on intravenous injection. The results obtained by Takahashi (1915) and by Watanabe (1954) however, indicate that entry into the vascular system is not synonymous with metastasis and that some tumours lack the ability to form secondaries even when definitely present in the venous circulation. Human studies by Engell (1955) give similar indications. The results of the present experiments are in accord with this point of view.

MATERIALS AND METHODS

Young, mature Sprague-Dawley rats, both male and female, were used in all the experiments. They were housed in metal cages and maintained on a diet of fox cubes. Space limitations necessitated keeping the animals in groups of about six, and cannibalism, pneumonia and regression of tumours unfortunately reduced the effective numbers in the experiments considerably. Only those rats that could be autopsied completely, and which had growing tumours at the time of death, are included in this report.

The tumours were obtained through the kindness of Dr. Millar and Dr. Noble, and consisted of eight different sarcomata, all of which arose in the same transplantable mammary fibroadenoma. Some of these have been described by these workers (Millar and Noble, 1954*a* and 1954*b*). The following tumours were used :

52 FM, 47 FM.	Designated as low grade fibrosarcomata. Relatively fibrous tumours of moderate cellularity. 47 FM showed considerable boney metaplasia. (Fig. 1 and 3).
45 FM.	Designated a liposarcoma. Composed of mixed fibrous and fatty tissue. (Fig. 2).
41 FM, 42 FM.	Designated moderate grade fibrosarcomata. (Fig. 9 and 11.)
35 FM, 17 FM, 53 FM.	Designated high grade fibrosarcomata. Relatively soft and cellular. (Fig. 13, 15 and 17.)

Technique of transplantation

All operations were performed under ether anaesthesia. Tumour for transplantation was removed under sterile conditions from subcutaneous grafts and diced with scalpels while bathed in saline.

Subcutaneous transplants were carried out by making a small incision in the right anterior axilla and inserting a 5 mm. fragment with the aid of forceps. The incision was closed with one or two clips.

Intramuscular transplants were accomplished by incising the ventral muscles of the right thigh and introducing a 5 mm. fragment. The muscle incision was closed with black silk, and the skin was closed with one or two clips.

Massaged tumours : Subcutaneous tumours were transplanted in the way described above. When tumours reached approximately 2 cm. in diameter they were manually massaged, using considerable force in the case of the more fibrous tumours. This was carried out daily for four or five days.

Repeated subtotal removals : Subcutaneous tumours were allowed to reach approximately 4 cm. in diameter. Where ulceration and haemorrhage occurred the removal was performed earlier. The tumour was dissected free and removed, leaving a 5–10 mm. flat fragment attached to the underlying tissue. The removed tumour was weighed and fixed in formalin : the skin was closed with a row of clips.

Intravenous injections were made into the tail vein without anaesthesia. Approximately two grams of tumour, free from necrosis, were immersed in a balanced salt solution and subjected to prolonged mincing with scalpel blades. The turbid suspension of tiny fragments was then gently mashed in a glass cylinder with a loosely fitting plunger. The supernatant fluid contained single cells and clumps which the addition of trypan blue (Zeidman, McCutcheon and Coman, 1950) indicated were about 15 per cent viable. Absolute cell counts were not done routinely, although an effort was made to produce roughly comparable suspensions. Each rat received 0·2 ml. of the suspension. Preliminary trials showed that incorporation of grossly necrotic tumour resulted in rapid death of the animal : this was avoided by using care in the choice of tumour fragments.

All rats bearing grafted tumours were kept until dead or moribund. Their tumours were weighed, and a complete autopsy was done. Rats injected intravenously were kept for approximately seven weeks : by this time nearly all the tumours found could be seen with the naked eye. All tumours were examined histologically ; sections were also made of each lobe of all lungs, and of any other organ showing gross metastases.

Of the eight tumour sublines used, all were studied as ordinary subcutaneous grafts, repeated subtotal removals, and intravenous injections. Five of the tumours were also studied as intramuscular transplants and subcutaneous grafts with massage of the tumour (Table I.)

RESULTS

The results from the standpoint of metastases formation are outlined in Table I. The essential features can be summarized as follows ;

1. Spontaneous metastases occurred in at least one instance from six of the eight tumour sublines studied. Ordinary subcutaneous grafts yielded metastases in three of the eight strains, and intramuscular grafts produced metastases in

TABLE I.—*Number of Rats with Metastases*

Tumour line.	Ordinary subcutaneous grafts.	Intramuscular grafts.	Subcutaneous massaged.	Subcutaneous with repeated subtotal removal.	Total with spontaneous metastases.	Intravenous injections.
52 FM	0/8	—	—	0/10	0/18	0/8
45 FM	0/10	—	—	0/8	0/18	0/8
47 FM	0/7	—	—	2/9	2/16 (12·5%)	2/22 (9·1%)
35 FM	3/7	2/7	1/10	3/13	9/37 (24·3%)	4/11 (36·4%)
42 FM	0/6	1/4	1/6	0/16	2/32 (6·3%)	0/15 (0%)
41 FM	0/8	0/7	0/6	1/10	1/31 (3·2%)	0/8 (0%)
17 FM	2/7	2/5	1/6	0/4	5/22 (22·7%)	3/12 (25%)
53 FM	1/6	4/7	1/7	5/8	11/28 (39·3%)	5/12 (41·7%)
Total	6/34 (17·6%)	9/30 (30%)	4/35 (11·4%)	9/51 (17·6%)	28/150 (18·7%)	12/58 (20·7%)

four of the five strains grafted in this manner. Two sublines failed to metastasize under any condition studied. When metastases occurred, they were always present in the lungs ; a few showed metastases to other organs in addition. (Fig. 3–18).

2. Neither repeated subtotal removal nor massage of the tumour increased the overall incidence of metastases. Two sublines, however (47 FM and 41 FM) yielded spontaneous metastases only after repeated removals. (Fig. 3–10.)

3. Intravenous injection of tumour cells produced lung metastases only in the case of those tumours which were capable of spontaneous metastases. The incidence of metastases from this technique roughly paralleled, with each subline and as a whole, the incidence of spontaneous metastases. This correlation should probably be regarded as fortuitous since the amount of tumour injected was purely arbitrary, and was only roughly equal among the various strains.

TABLE II.—*Primary Tumour Weight, Life Span and Tumour Growth Rate under Varying Environmental Conditions*

	Average weight of primary tumour in grams.		Average life span of tumour-bearing rat in weeks.		Average growth rate* of tumour in grams per week.	
	Rats without metastases.	Rats with metastases.	Rats without metastases.	Rats with metastases.	Rats without metastases.	Rats with metastases.
Ordinary subcutaneous grafts	193±20·9	128±29·4	8·0±0·46	6·3±1·22	21·5±2·2	19·0±3·6
Intramuscular grafts	171±15·5	191±31·8	10·7±0·85	13·3±4·43	15·9±1·2	15·6±1·8
Subcutaneous grafts —massaged	127±11·1	196±42·5	7·0±0·69	11·5±2·35	16·5±1·1	16·4±1·6
Subcutaneous grafts with repeated subtotal removal	135±15·0	170±24·7	14·1±1·17	21·1±2·68	10·7±0·8	10·3±1·9
Average of all rats	152	171	10·2	14·0	15·5	14·7

* Calculated from growth rates of individual tumours.

In Table II an analysis of the final tumour size, life span and growth rates is given for those tumours which were studied under all four conditions, and a

comparison is made between tumours which metastasized and those which remained localized. The data indicate that :

(i) Subcutaneous tumours grew more rapidly and killed the animal at an earlier date than did intramuscular grafts.

(ii) The growth rate of the tumours which metastasized was not significantly different from that of those which did not. On an average it would appear that there was slightly longer survival and a greater final tumour size where metastases occurred. The individual variations were, however, so great that the differences are not statistically significant. In fact, one of the most striking points noted throughout the experiment was, in spite of fairly uniform growth rate, the tremendous variation in individual survival time, and the lack of correlation between life span and metastasis formation.

TABLE III.—*Primary Tumour Weight, Life Span and Tumour Growth Rates of Three Tumour Sublines*

Tumour.	Average weight of primary tumour in grams.		Average life span of tumour-bearing rat in weeks.		Average growth rate* of tumour in grams per week.	
	Rats without metastases.	Rats with metastases.	Rats without metastases.	Rats with metastases.	Rats without metastases.	Rats with metastases.
35 FM	96± 9·7	103±19·0	6·0±0·8	7·6±1·7	19·8±2·1	16·6±2·7
17 FM	129±18·7	178±28·4	10·8±1·5	11·4±0·7	12·7±1·8	15·9±2·5
53 FM	194±27·3	233±20·0	13 ±1·5	18·5±1·5	16·8±2·2	13·1±1·7

* Calculated from growth rates of individual tumours.

DISCUSSION

The studies described were commenced in the belief that the tumours used would not metastasize spontaneously under the usual transplantation procedures, and were carried out in the hope of finding under what circumstances metastases could be made to occur. The demonstration that an appreciable number metastasized from simple subcutaneous and intramuscular grafts was unexpected and is difficult to reconcile with the findings of previous workers. Little importance can be ascribed to the fact that some of the tumours used have been propagated for a considerable time, since the 53 FM tumour, which yielded the highest rate of metastases, arose only a few weeks before the experiments were performed. A very careful search was made for metastases in the present study, but even a cursory examination of the lungs would have given essentially the same results as far as metastatic frequency is concerned. The experiments do establish beyond doubt that some examples of this particular type of sarcoma are true malignant neoplasms in behaviour as well as in appearance.

From the present study there is little evidence that massage or repeated subtotal ablation of a tumour affects appreciably the incidence of metastases. Possibly these effects are more readily demonstrated with tumours more prone to metastasize ; a definite trend might have been established with the present group of tumours had more animals been used. Differences in the vascularity and consistency of individual tumour types may also account for the discrepancy between the results of the present study and those of previous workers. While

repeated removals failed to increase the overall incidence of metastases, two strains metastasized only after this procedure, and some evidence of increasing tumour anaplasia during the life span of the host was seen (Fig. 10). The general failure of this procedure may have been due to insufficient prolongation of life; with each succeeding removal the tumours became increasingly difficult to dissect free, and operative mortality rose after two or three removals.

The results indicate that, with this particular group of tumours, the occurrence of metastases is not dependent solely upon vascular invasion and embolization; intravenous injections produced tumours only with those subline tumours capable of spontaneous metastasis. While this seems completely at variance with the findings of some workers (Weil, 1913; Andervont and Shimkin, 1941), it seems possible that different types of tumours may vary in this regard. Some tumours may principally lack the ability to invade vessels and yet be quite capable of growth when artificially disseminated, while others may be more notably deficient in their powers of forming new tumour foci from embolic origin. The failure to influence the incidence of metastases by mechanical interference or intravenous injection in the present study does not establish that factors necessary for metastasis reside solely in the tumour cells.

Although the data show no significant relation between metastasis formation and final tumour size, life span or growth rate, the extensive work of Wood and his associates (1954) shows that a large number of animals may be necessary to establish a significant trend. The present data are based on a relatively small number of animals, and the results show too much individual variation. They therefore probably should not be considered to be evidence against the conclusions of these workers.

Three characteristics of the tumours under investigation deserve consideration:

1. The time required for spontaneous metastases to develop is variable and unpredictable in spite of the fact that venous invasion is common.

EXPLANATION OF PLATES

All sections were stained with Haematoxylin and eosin.

FIG. 1.—52 FM tumour showing fibrous structure. No metastases occurred from this subline. × 80.

FIG. 2.—45 FM tumour showing immature fat cells. No metastases were observed. × 80.

FIG. 3.—47 FM tumour showing fibrous tissue and early bone formation. Spontaneous metastases occurred after repeated subtotal removal. × 80.

FIG. 4, 5, 6, 7 and 8.—Spontaneous metastases from 47 FM tumour after repeated subtotal removal. Fig. 4, 5 and 6 show pulmonary metastasis composed of fibrous tissue and bone. Fig. 6 (from a decalcified section) demonstrates the extreme degree of bone formation encountered in some pulmonary nodules. Metastasis to the kidney is seen in Fig. 7, and to the myocardium (left ventricle) in Fig. 8. Fig. 4 × 50. Fig. 5, 6, 7 and 8. × 80.

FIG. 9.—41 FM tumour showing early venous invasion. This tumour metastasized only after repeated subtotal removal. × 80.

FIG. 10.—Spontaneous lung metastasis from 41 FM tumour. The cells appear more pleomorphic and anaplastic than those seen in the primary tumours. × 80.

FIG. 11.—42 FM tumour. × 80.

FIG. 12.—Spontaneous lung metastasis from 42 FM tumour. × 80.

FIG. 13.—35 FM tumour. × 80.

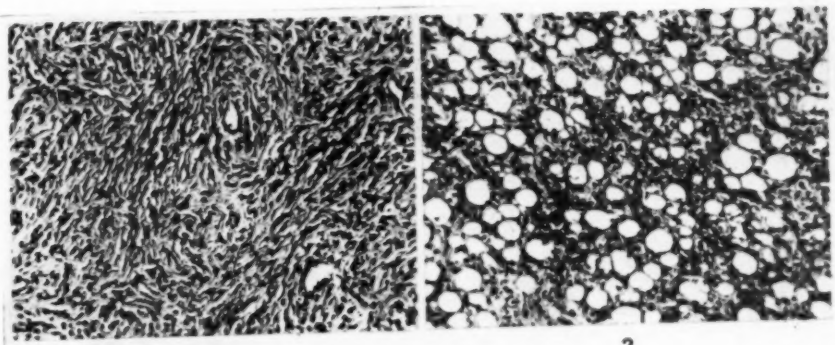
FIG. 14.—Spontaneous lung metastasis from 35 FM tumour. × 80.

FIG. 15.—17 FM tumour. × 80.

FIG. 16.—Spontaneous lung metastasis from 17 FM tumour. × 80.

FIG. 17.—53 FM tumour. × 80.

FIG. 18.—Spontaneous lung metastasis from 53 FM tumour. × 50.



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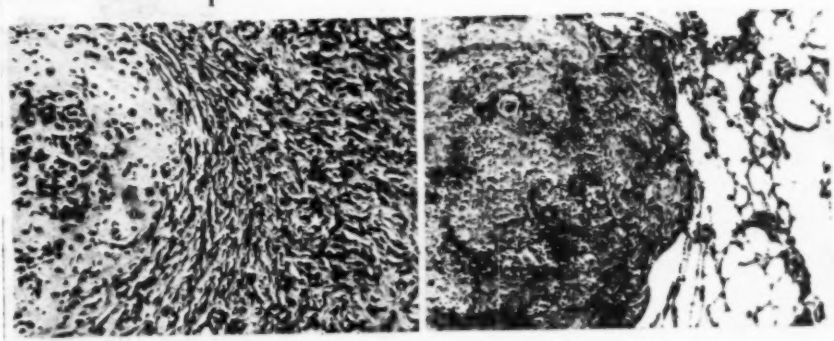

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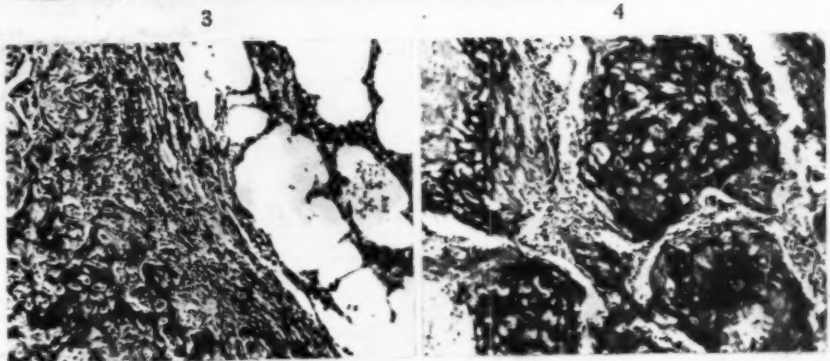

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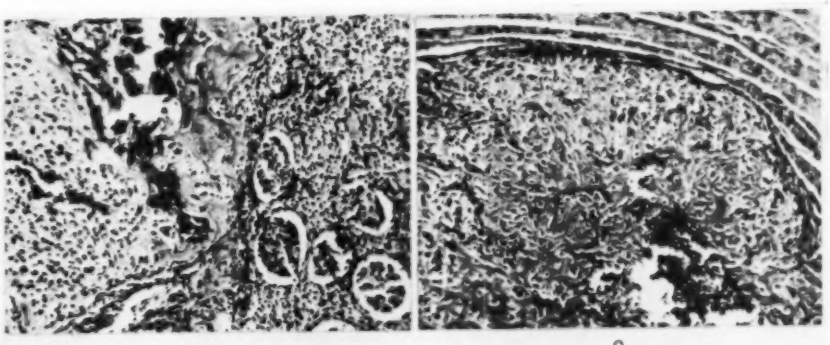

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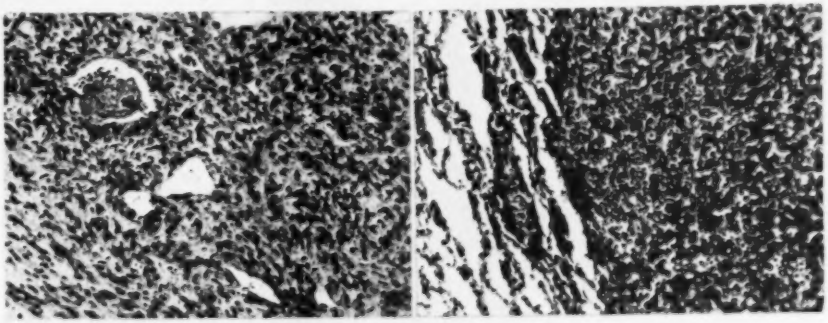

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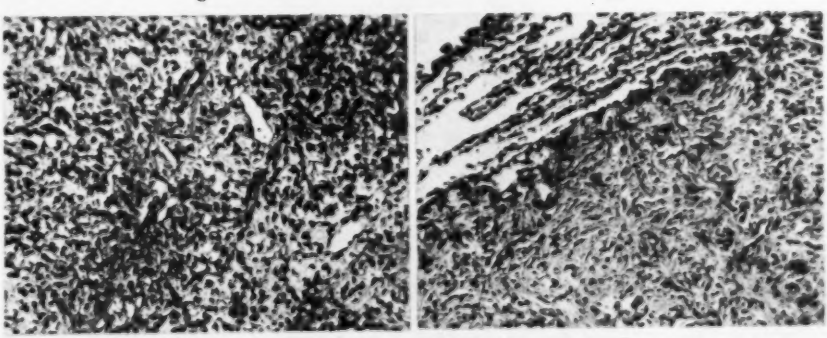

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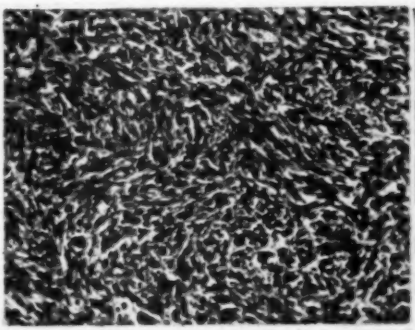

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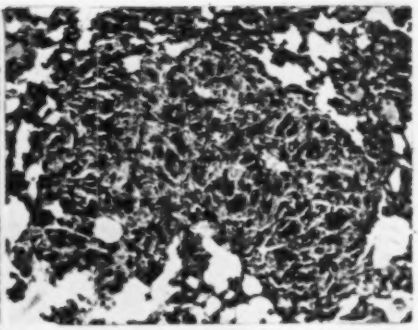

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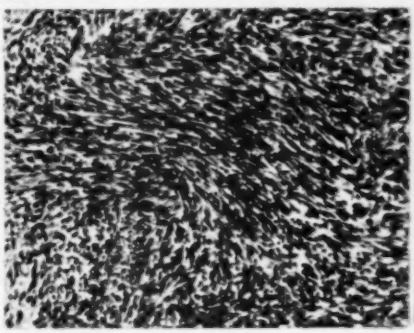

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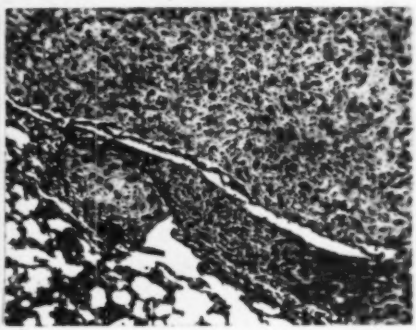

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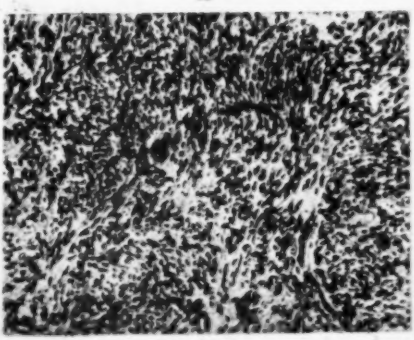

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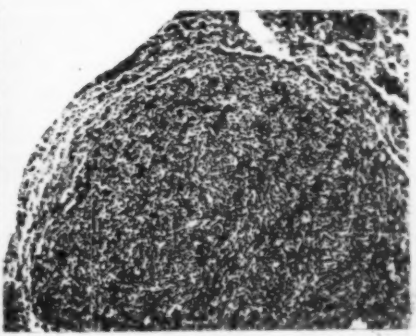

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2. Intravenous inoculation is not uniformly successful in producing lung tumours.

3. A large amount of tissue is apparently needed for transplantation of the tumours.

It has been customary to transplant this tumour by means of relatively large fragments, and unpublished trials by myself indicate that very large numbers of cells are, in fact, required for successful takes. All three of these characteristics might be explained on the basis of a diverse cell population within the tumour. Some very malignant tumours, such as ascites tumours and lymphosarcomas, are transplantable with only a single cell (Furth and Kahn, 1937 ; Hauschka, 1953). It seems obvious that such tumours must be composed almost entirely of transplantable cells, each capable of independent growth. In contrast, tumours such as those used in the present study may contain only a small proportion of such cells. Thus, uniform success in transplantation might require a large amount of tissue, and, of all the cells gaining entrance into the circulation, either spontaneously or artificially, only occasional clumps might be successful in producing metastases. The unpredictable behaviour of some human tumours might have the same explanation. Death of tumour emboli in small vessels has been observed by innumerable workers with both human and experimental tumours ; likewise, the concept of a heterogeneous tumour cell population is not an original one. Nonetheless, lack of uniformity of transplantability within a given tumour population might be investigated more extensively as an explanation of why some tumours fail to show a consistent pattern of spread and produce occasional solitary metastases in spite of obvious venous invasion. The proportion of cells capable of independent growth may be the determining factor in such behaviour.

SUMMARY

A group of sarcomata arising from a single transplantable rat fibroadenoma has been studied under a variety of conditions in order to determine under what circumstances metastases would occur. Contrary to the reports of previous workers, an appreciable number of these tumours are capable of spontaneous metastasis and should be considered true malignant tumours. Massage of the transplanted tumour did not increase the frequency of metastases. While repeated subtotal removal did not increase the overall incidence of metastases, it produced spontaneous metastases in two sublines which did not occur under any other circumstances. Artificial metastasis by intravenous inoculation was successful only with those sublines which were capable of spontaneous metastasis. While the growth rate of the tumours was fairly uniform, there was great variation in the survival time, final tumour size, and time required to produce metastases. Possible explanations for some of the results have been discussed.

This work was supported by a grant-in-aid from the National Cancer Institute of Canada.

I am grateful to Mrs. Susan Davis, Mrs. Grace Strickland and Miss Bette Byrns for technical assistance. My thanks are due to Miss Lizetta Nason for taking the photographs and to Dr. Margaret Owchar of the Manitoba Cancer Institute for evaluating the statistical data.

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THE PROLIFERATIVE CAPACITY OF THE LINING EPITHELIUM OF THE ANAL CANAL IN MICE

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In this communication the results are recorded of implanting pellets of paraffin wax, containing methylcholanthrene, beneath the muco-cutaneous junction of the anal canal in mice. Since these experiments were designed to study the carcinogenic effect of methylcholanthrene on the mucous and squamous epithelium entering into the junction, results have been compared with those of similarly implanting control pellets containing no methylcholanthrene. In addition, the proliferation of the epithelium adjacent to the muco-cutaneous junction has been studied after the resection of part of the area of the anal lining in which the junction lies.

MATERIALS AND METHODS

The mice used were from inbred laboratory stock but were of no particular strain. Fully-grown adults of both sexes were used but no differences due to sex were observed.

The implantation of paraffin pellets

The pellets used were made of paraffin wax, melting point 56° C., and were about 2 mm. in diameter and less than 1 mm. thick. Methylcholanthrene, when present, was in a concentration of 5 per cent. The pellets were implanted into animals that had been anaesthetised with ether and then tied down on their backs when, by suitable retraction at the anal verge, the dorsal wall of the anal canal was exposed up to and beyond the muco-cutaneous junction. A small incision was then made at the anal verge, the pellet insinuated beneath the squamous epithelium of the anal canal, and pushed cranially until it lay beneath the muco-cutaneous junction and thus beneath both squamous and mucous epithelium. Retention of the pellet in this position was aided by closing the wound at the anal verge with a fine silk stitch. Subsequently, at various intervals, the effect on the anal lining of these pellets, with and without methylcholanthrene, was compared by making longitudinal sections of the anal canal.

Excision of the anal lining in the region of the muco-cutaneous junction

As before, the dorsal wall of the anal canal was exposed in anaesthetised mice and from this aspect an area of the lining resected. The area extended 1–2 mm., both cranially and caudally, from the muco-cutaneous junction and about one-third of the circumference of the anal lining was removed. Care was taken to preserve intact the underlying muscular structures but some damage to them could not always be avoided. The consequences of such lesions were subsequently studied in longitudinal sections.

OBSERVATIONS

The normal anatomy of the anal canal

Many experiments resulted in an extension of the squamous epithelium cranial to the normal muco-cutaneous junction and thus into regions normally covered by mucous glands. This extension, although easily identified macroscopically, could only be assessed quantitatively in longitudinal sections by relating the muco-cutaneous junction to underlying structures. In the normal anal canal this junction lies at the level of the cranial border of an external sphincter of striated muscle which is separated from other muscle elements of the anal wall by the anal sebaceous glands and associated hair follicles. The transition from squamous to mucous epithelium is abrupt. Further, it should be noted that between the orifices of the anal glands and the muco-cutaneous junction, the squamous epithelium is devoid of both hair follicles and sebaceous glands (Fig. 1).

Following implantation of paraffin pellets

To study the effect of methylcholanthrene on the anal lining changes were compared in experiments when the implanted pellets contained this substance and when they did not. Such comparisons were possible under two sets of circumstances; firstly when the pellets remained in position beneath the muco-cutaneous junction, and, secondly, when, after remaining in this position for an arbitrarily chosen minimum period of 21 days, the pellets were discharged.

When the pellets were retained.—No significant differences were found when the pellets contained 5 per cent methylcholanthrene and when they did not. The effects found in both experiments are seen in Fig. 2, which illustrates an experiment in which a methylcholanthrene-containing pellet was implanted 83 days previously. The pellet lies beneath both the squamous and mucous epithelium at the muco-cutaneous junction. The mucous epithelium, apart from some stretching, shows no significant ch[illegible] certain[illegible]one to suggest any abnormal proliferation. The significance of [illegible]ive [illegible]is, however, considerably diminished for it was not possible [illegible].rficial to the muscularis mucosae which therefore separate[illegible]ene in the pellet from the dividing cells in the bases of t[illegible] the other hand, in the vicinity of the pellets, changes are found in the [illegible]us epithelium, but they are essentially the same whether or not it contains methylcholanthrene. These changes are also shown in Fig. 2. Immediately adjacent to the muco-cutaneous junction the squamous epithelium has become thickened with some downgrowth of epithelium into the underlying fibrous tissue but the downgrowth goes no deeper than this and does not reach the underlying muscle layers. Around the pellet is the tissue reaction often found around foreign bodies containing paraffin (cf. Gabriel, Dukes and Bussey, 1951), and this occurs to an equal extent when the pellet contains methylcholanthrene and when it does not. It can be added that such a reaction may shield adjacent cells from the action of methylcholanthrene and thereby account, in part, for the lack of carcinogenic effect both on the mucous and the squamous epithelium. In addition, no evidence of neoplastic transformation could be observed in the connective tissue cells in the neighbourhood of those pellets which contained methylcholanthrene.

When pellets were extruded 21 days or more after implantation.—Such extrusion took place through the mucous membrane immediately cranial to the muco-cutaneous junction. A breach was therefore created in the mucous membrane which became filled by a cranial extension of the squamous part of the lining of the anal canal. Superficially the magnitude of this extension could be determined by macroscopic examination of the lining of the anal canal after its removal. Thereby it was found that, neither in magnitude nor in frequency did this extension differ whether or not the extruded pellet had contained methylcholanthrene. In each case it occurred in about 75 per cent of experiments. In Fig. 3 such a cranial extension of squamous epithelium is shown in longitudinal section; it can be seen to pass beyond the normal level of the muco-cutaneous junction at the cranial border of the anal glands and also of the external sphincter of striated muscle. The figure also demonstrates that, in addition to this superficial cranial extension, the squamous epithelium undergoes other proliferations. These additional proliferations, which are regularly found whether the extruded pellet contained methylcholanthrene or not, may take a variety of forms and, in this respect alone, do results with methylcholanthrene pellets differ from controls. The additional proliferations have therefore been arbitrarily classified into a number of groups and the frequency with which each occurs has been determined in experiments examined 80–180 days after the implantation of paraffin pellets at the muco-cutaneous junction and their subsequent extrusion. The groups chosen were as follows:

(*a*) Downgrowth of epithelium into underlying connective tissue but not deeper (Fig. 3).

(*b*) Downgrowth of squamous epithelium penetrating the muscle coats of the anal canal (Fig. 4).

(*c*) Papillomatous formations on the surface of the cranial extension of the squamous epithelium (Fig. 5).

(*d*) The formation of macroscopic tumours (Fig. 6). This figure shows the cranio-caudal extent of the largest of such tumours observed which, in this case, extended around the anal canal for more than half its circumference. In such tumours it was necessary to consider the possibility of malignant change but this appears to be unlikely since metastases were not found. Also, tumours of this type were well encapsulated and the component strands of squamous epithelium were regular in outline and in no way anaplastic (Fig. 6).

In Table I the frequency with which proliferations, so classified, were found is compared when the paraffin pellets contained methylcholanthrene and when they did not.

Following resection of the anal lining at the muco-cutaneous junction

Such resections, performed as already described, result in an extension cranially of squamous epithelium into the area normally covered by mucous glands. Since such extensions were found to be comparable with those following the extrusion of implanted paraffin pellets, they have been studied to determine the extent to which they, also, are associated with additional proliferations of the sort listed in Table I.

TABLE I.—*Proliferation of Squamous Epithelium at the Muco-cutaneous Junction Following the Implantation Beneath it of Paraffin Pellets and their Subsequent Extrusion After a Minimum Period of 21 Days*

Group	Pellets containing methylcholanthrene		Pellets without methylcholanthrene	
	No.	Per cent	No.	Per cent
a	44	67	21	75
b	8	12	7	25
c	8	12	—	—
d	6	9	—	—
Total	66		28	

The proliferations recorded were observed after 80–180 days, and, in every case, were additional to a superficial cranial extension of the squamous epithelium which occurred, to an equal extent when the pellets contained 5 per cent methylcholanthrene and when they did not. The groups *a*, *b*, *c*, *d*, into which the proliferations have been classified, are described in the text.

Within a week of performing the resection the reaction can be seen to differ in the persisting edges of the squamous and mucous epithelium. Proliferative changes in the latter are slight and confined to a scanty outgrowth, over the denuded area, of a single layer of epithelium. No further extension of the mucous membrane occurs and the area from which this membrane was resected becomes progressively covered by a cranial extension of squamous epithelium. This process becomes complete in 20–30 days (Fig. 7) and at the new junction of squamous and mucous epithelium the transition is as abrupt as in the normal. The early stages of this extension of squamous epithelium can be seen by the end of the second week, when in addition, there is a downgrowth of squamous epithelium into underlying tissue (Fig. 8). Such downgrowths were found, at this stage, in more than half of the resections performed. In a number of cases such additional proliferations persist after the cranial extension of squamous epithelium meets the persisting mucous membrane. Among these, in 2 of 20 specimens examined 100–150 days after the resection, the epithelial strands had penetrated into the muscle layers (Fig. 9) producing appearances similar to those of group *b*, (Table I and above) which were observed after the extrusion of implanted paraffin pellets not only when they contained methylcholanthrene but also when they did not.

DISCUSSION

The results of resecting areas of anal lining containing the muco-cutaneous junction demonstrate so great a difference in the proliferative capacity of the squamous and mucous epithelium that the defect in the lining of the anal canal becomes completely covered by the former. This is in accordance with results previously recorded (O'Connor, 1954, 1956) when localised areas of mucosa were destroyed or resected in more proximal parts of the rectum and colon. No capacity of the mucosa to extend over denuded areas could be demonstrated. It seems reasonable to conclude therefore that the topographical stability of the muco-cutaneous junction depends on restraint of the proliferative capacity of the squamous epithelium due to its contact with the mucous. Evidence of such a restraint, may be seen in the abrupt transition, from squamous to mucous epithelium, which occurs both at the normal muco-cutaneous junction and at junctions experimentally produced at abnormal levels.

It is possible therefore to suggest, that if this contact is weakened by mechanical means, an excess of squamous proliferative capacity may take place at the muco-cutaneous junction. Such a weakening may occur as the result of the tension due to paraffin pellets implanted at the junction, and such is the explanation offered for the proliferation of squamous epithelium found when implanted pellets remain *in situ* (cf. Fig. 2). Such an explantion accounts, in addition, for the similarity of proliferation when, under such circumstances, the pellets contain methylcholanthrene and when they do not.

When the implanted pellets are subsequently extruded, a breach in the mucous membrane at the muco-cutaneous junction can be assumed to occur, into which squamous epithelium extends, independently of the presence or absence of methylcholanthrene in the implanted and extruded pellets. It is more doubtful however, whether the qualitative differences noted in the additional proliferations associated with this extension, recorded in Table I, are to be ascribed to the carcinogenic action of methylcholanthrene when it is present in the pellets. In the first place, the choice of the arbitrary and crude divisions into which the types of proliferation have been divided makes the data unsuitable for statistical analysis so that tests of the significance of the difference cannot be applied. Secondly, groups *a* and *b* not only occur when pellets contain no methylcholanthrene but they also occur, in a comparable form, following resection of areas of the anal lining which include the muco-cutaneous junction. Thirdly, neither groups *c* nor *d*, which were recorded only when the pellets contained methylcholanthrene, can, by conventional histological standards, be called malignant. Finally, the proliferations listed in Table I occur in squamous epithelium which has no hair follicles. Since the important role of these in chemical carcinogenesis has been stressed by Wolbach (1951), Whitely and Ghadially (1952) and by Liang and Cowdry (1955), it may be considered unlikely that methylcholanthrene exerts a carcinogenic effect when applied to the squamous epithelium of the anal canal.

In the case of the mucous epithelium at the anal muco-cutaneous junction, no evidence has been found to warrant any consideration of the possibility that methylcholanthrene applied as described, has a carcinogenic effect. Although, as already pointed out, in the present experiments, the contact between the epithelium and methylcholanthrene may be inadequate for carcinogenesis, these negative results correspond to the considerable amount of evidence collected by Laurens and Bacon (1952) showing that cells of mucous membrane of the large intestine are refractory to chemical carcinogens, and also to the findings that, under natural conditions, neoplasms in mice are rare in this region (Wells, Slye and Holmes, 1938).

SUMMARY

Pellets of paraffin wax containing methylcholanthrene have been implanted beneath the muco-cutaneous junction of the anal canal of mice.

No effect, attributable to the carcinogen, could be demonstrated on the mucous epithelium entering into the junction.

Observations were made on a number of experiments in which, after remaining *in situ* for 3 weeks or more, pellets were extruded. Under such circumstances, proliferation of the squamous epithelium was more varied and extensive when the

pellets contained methylcholanthrene than with control pellets. It was not possible however to establish that these differences were statistically significant.

These proliferations have been compared with those that follow resection of areas of the anal lining containing the muco-cutaneous junction. Under both circumstances the proliferations were sufficiently similar to make impossible the certain exclusion that all proliferations of squamous epithelium which follow the implantation of methylcholanthrene in paraffin pellets at the muco-cutaneous junction are non-specific in origin.

The difference in proliferative capacity of the squamous and mucous epithelium at the muco-cutaneous junction is so great that the topographical stability of the junction is ascribed to a restraint of proliferation exerted on the squamous epithelium by its contact with the mucous.

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EXPLANATION OF PLATES

FIG. 1.—Longitudinal section of normal anal canal showing relationship of the muco-cutaneous junction to the anal glands and the external sphincter of striated muscle. × 27.

FIG. 2.—Anal canal 83 days after the implantation of a paraffin pellet containing methylcholanthrene. Note the proliferation of squamous epithelium at muco-cutaneous junction. × 18.

FIG. 3.—Anal canal 141 days after the implantation at the muco-cutaneous junction and subsequent extrusion of a paraffin pellet containing methylcholanthrene. The squamous epithelium has extended cranially and there is also some proliferation into the underlying connective tissue. × 33.

FIG. 4.—Anal canal 126 days after the implantation at the muco-cutaneous junction and the subsequent extrusion of a paraffin pellet containing no methylcholanthrene. Proliferation of squamous epithelium with penetration into the underlying muscle coats. × 22.

FIG. 5.—Anal canal 148 days after the implantation at the muco-cutaneous junction and the subsequent extrusion of a paraffin pellet containing methylcholanthrene. Papillomatous proliferation of the squamous epithelium. × 22.

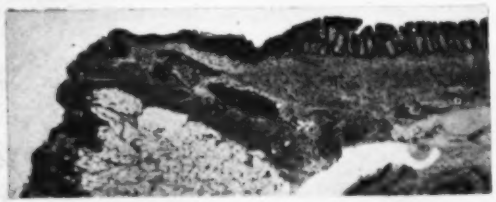
FIG. 6.—Anal canal 150 days after the implantation at the muco-cutaneous junction and the subsequent extrusion of a paraffin pellet containing methylcholanthrene. Development of a large tumour of squamous epithelium. The tumour is well encapsulated and there is no anaplasia of the constituent epithelial strands. × 14.

FIG. 7.—Anal canal 34 days after the resection of an area of its lining containing the muco-cutaneous junction. Cranial growth of the squamous epithelium into the area from which the mucous epithelium was removed. Specimen fixed in formalin : anal verge to the left. × 8.

FIG. 8.—Anal canal 10 days after the resection of an area of its lining containing the muco-cutaneous junction. Extension cranially of squamous epithelium with proliferation extending into the underlying connective tissue. × 31.

FIG. 9.—Anal canal 203 days after the resection of an area of its lining containing the muco-cutaneous junction. Extension cranially of the squamous epithelium with penetration into the underlying muscle coats. × 25

Vol. X, No. 4.
BRITISH JOURNAL OF CANCER.



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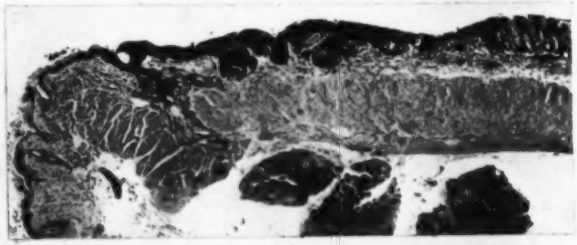

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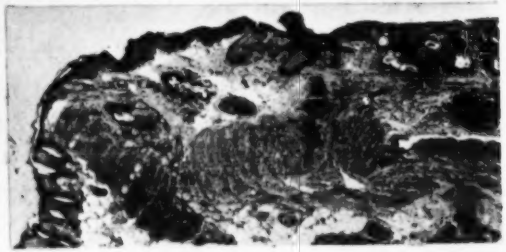

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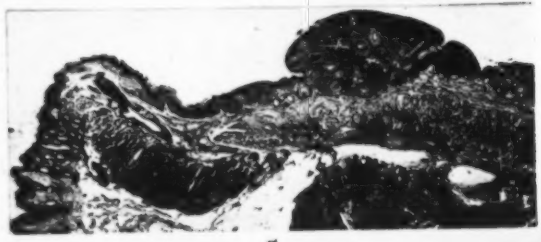

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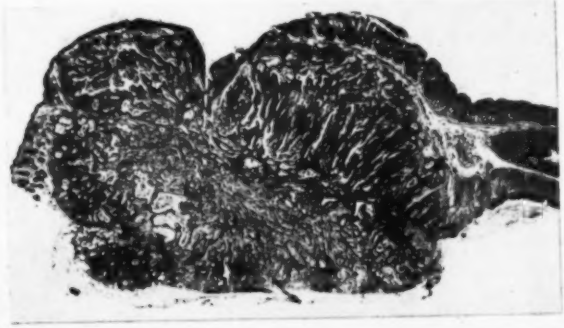

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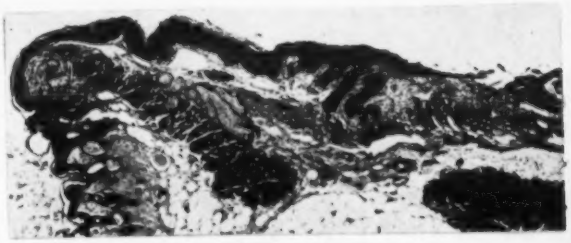

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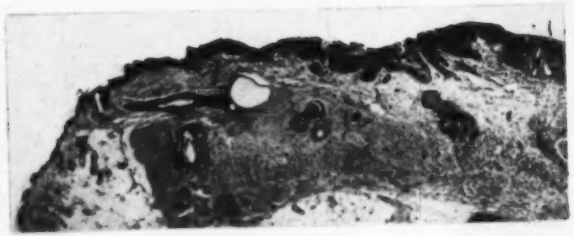

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TRACER STUDIES OF POTENTIAL RADIOSENSITIZING AGENTS

TETRASODIUM 2-METHYL-3-^{82}Br-BROMO-1 : 4-NAPTHOHYDROQUINONE DIPHOSPHATE AND TETRASODIUM 2 : 3-DIMETHYL-5 : 6-DI^{131}I-IODO-1 : 4-BENZOHYDROQUINONE DIPHOSPHATE

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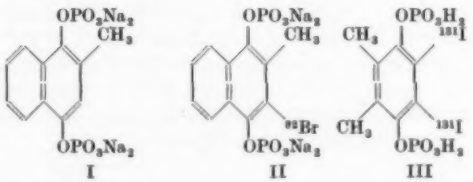
THIS paper continues our studies (Marrian and Maxwell, 1956) of potential radiosensitizers, compounds which, it is hoped, might selectively concentrate in fast growing tissues and there accentuate the effects of ionising radiations. There are good reasons for believing that tetrasodium 2-methyl-1 : 4-naphthohydroquinone diphosphate (Synkavit) (I) can accumulate in tumour tissue for short periods after intravenous injection (Mitchell, 1955 ; Marrian and Maxwell, 1956) and we felt that related compounds carrying a γ-emitting isotope might combine radio-sensitization with, possibly, a useful dose of localised radiation. ^{82}Br and ^{131}I seemed the most useful isotopes for this purpose and this paper records some observations on the metabolism of tetrasodium 2-methyl-3-^{82}Br-bromo-1 : 4-naphthohydroquinone diphosphate (II) and also of 2 : 3-dimethyl-5 : 6-di-^{131}I-iodo-1 : 4-benzohydroquinone diphosphate (III). The antimitotic properties of the former compound have been established (Friedmann, Marrian and Simon-Reuss, 1952) and although the latter is inactive by the same test (Simon-Reuss, unpublished) it seems to be one of the few hydroquinone diphosphates in which labelling with ^{131}I is simple. Studies of its metabolism were therefore worthwhile, and served to confirm the lability of halogen in a substituted hydroquinone ring.



METHODS AND MATERIALS

Counting.—γ-emission from the halogens was the basis of detection. The simple technique of placing samples of the organs under a shielded counter could not be used since variations in the thickness of the organs would vary the sample-counter distance, leading to considerable counting errors. This type of error was reduced by locating the sample on a thin aluminium tray midway between two G-10-Pb glass-covered Geiger-Muller tubes in copper sheaths placed 10 cm. apart in a castle of 2 inch lead and connected in parallel to the same scaler. The

sensitivity of the arrangement was about 9000 c.p.m./μC ^{82}Br. Samples and backgrounds were counted to a standard error of 5 per cent or less.

Solutions for injection

The synthesis of both compounds (II and III) has been reported (Andrews, Marrian and Maxwell, 1956). The hydroscopic salt (II) was dissolved in 0·9 per cent saline and the concentration determined by ultraviolet spectroscopy (absorption maxima at 232mμ. (M = 70,000) and 290 mμ. (M = 5,800) in 0·01N-hydrochloric acid). A solution of the compound ran as a single spot on paper in saturated ammonium sulphate (80 vols.), 1 M-sodium acetate (20 vols.) and iso-propanol (2 vols.). Both counting and autoradiography of the dried paper strip showed that more than 95 per cent of the radioactivity was associated with the ultraviolet absorbing spot and that less than 0·6 per cent was present as bromide ion, as detected by spraying the dried paper with a saturated solution of fluorescein in 50 per cent ethanol and allowing to dry. Exposure to chlorine vapour caused red spots of bromofluoroscein to appear on a yellow background. The method is relatively insensitive, requiring about 100γ of bromide for detection, but it proved quite satisfactory for our requirements since as much carrier bromide as necessary could be added before separating.

The iodinated compound (III), was dissolved in 0·9 per cent saline and neutralised by addition of sodium hydroxide solution. The purity of the solution was determined by paper electrophoresis at pH 7 (Markham and Smith, 1952). This clearly separated the organic compound from iodide ion, the former being detected by ultraviolet photography and the latter by spraying with starch solution and exposure to chlorine vapour. No detectable radioactivity was associated with the area on the paper where carrier iodide appeared.

Ammonium ^{82}Br-bromide was obtained by neutron irradiation of ammonium bromide in the Harwell pile. It was dissolved in 0·9 per cent saline before injection.

Attempted exchange reactions

Tetrasodium 2-methyl-3-bromo-1 : 4-naphthohydroquinone diphosphate and ammonium ^{82}Br-bromide (9 moles excess) were incubated at 70° C. for 4 hours at pH 4, 6, 7 and 9 and the reaction mixtures examined by paper electrophoresis. No exchange greater than 0·5 per cent (the lower limit of detection) had occurred. When the reactants were present in molar ratio, the lower detection limit was 2·0 per cent but again no exchange could be detected.

Animal experiments

Rats carring a Walker carcinoma were used as previously described (Marrian and Maxwell, 1956). Immediately after the compound had been injected, and using the same syringe, aliquots of the dose were placed, in triplicate, on small pieces of filter paper on aluminium planchettes. The radioactivity of these planchettes when the organs were being counted was used to calculate the differential absorption ratio (D.A.R.), (Marrian and Maxwell, 1956). In most experiments with the bromo-compound, the time required to count the organ necessitated a decay correction, calculated from the known half-life of ^{82}Br-bromine (36 $\pm$ 0·2 hours).

RESULTS

The efficiency of the double counter in reducing errors due to sample thickness was deduced and confirmed as follows :

If A_0 is the measured activity of a sample at distance d from a counter and A_x is the activity of the same sample measured at distance x, then the error introduced by this displacement is

$$E = \frac{A_x - A_0}{A_0}$$

By the inverse square law, it can be shown that when a single counter is used the error is

$$E_1 = 2x/d + 3(x/d)^2$$

while if a sample is midway between two parallel counters, distance $2d$ apart, the error is

$$E_2 = 3(x/d)^2$$

This relation was verified by measuring the activity of a thin layer of silver ^{82}Br-bromide at various distances from the central tray with one and then both counters in operation. The errors E_1 and E_2 were determined and the results plotted in Fig. 1 which shows that the relations hold to a first approximation and that the error due to a sample thickness of 1·2 cm. would be 30 per cent using a single counter and 5 per cent using a double counter.

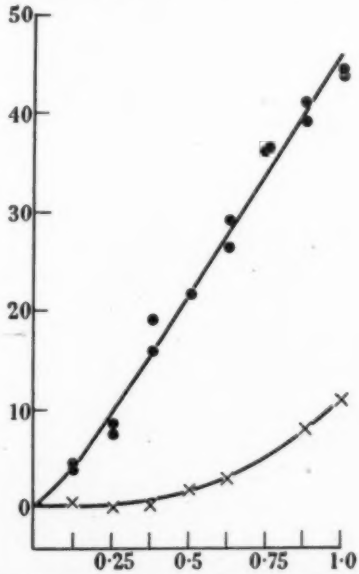


FIG. 1.—Reduction of the error in counting caused by varying sample thickness.
Upper curve : single counter. Lower curve : double counter.
Ordinate : Percentage error in the count.
Abscissa : Distance of the sample from the central plate in cm.

Table I records the variation of D.A.R. with time in the organs of tumour-bearing rats injected with 1 mg. (10·6 μC) of tetrasodium 2-methyl-3-^{82}Br-bromo-1 : 4-naphthohydroquinone diphosphate. The small intestines and kidney had an initial D.A.R. of about 1·5 which fell exponentially with time with a half-life of about 30 hours; liver, spleen and muscle showed little variation with time, the D.A.R. remaining between 0·5 and 1·0.

TABLE I.—*Differential Absorption Ratios ± Standard Errors.*

Time.	0·5 hours.	6 hours.	15 hours.	24 hours.	48 hours.
Tumour	2·1 ±·2	1·9 ±·3	1·6±·1	1·5 ±·1	1·6±·2
Muscle	0·55±·1	0·6 ±·15	0·4±·02	0·3 ±·1	—
Testis	1·0 ±·2	1·2 ±·1	1·4±·2	0·9 ±·07	1·1±·3
Liver	0·8 ±·1	0·9 ±·1	0·6±·03	0·4 ±·06	0·6±·15
Spleen	1·15±·05	0·9 ±·01	0·9±·01	0·75±·1	0·9±·1
Kidney	1·45±0·1	1·25±·15	0·9±·01	0·9 ±·1	0·9±·3
Stomach + Contents	3·5 ±·3	4·7 ±·8	3·4±·1	3·2 ±·3	5·0±·7
Small intestine	1·45±·01	1·1 ±·1	0·9±·1	0·7 ±·1	—
Brain	0·45±·04	0·5 ±·01	0·5±·03	0·4 ±·1	0·4±·1
	Percentage of injected dose				
Blood	14·0	12·4	12·1	9·0	6·6
Plasma	10·7	8·5	7·5	6·0	4·8
			(10 hours)		
Urine	—	2·7	17·0	28·0	37·0

The radioactivity associated with the blood also fell exponentially from about 15 per cent of the injected dose after 30 minutes. The half-life was about 40 hours and plasma accounted for some 67 per cent of the radioactivity of the whole blood.

The D.A.R. of the tumour was consistently higher than any of the above organs and was only exceeded by that of the stomach and contents. The radioactivity associated with the stomach, which will be discussed later in this paper, was not investigated in our initial work with this compound, but the early experiments serve to confirm the high tumour uptake compared with the other organs listed. Fig. 2 typifies the results of 3 experiments; the experimental points are where the lines change direction, each point being the mean determination on two rats. The individual determinations were within 5 per cent of the mean. Removing as much of the blood as possible before dissection and counting did not alter the general picture.

Radioactivity was slowly excreted in the urine, some 30–35 per cent of a 1 mg. (10·6 μC) dose appearing during 48 hours. Paper chromatography of samples from a rat given 5 mg. (II) intravenously indicated that most of the radioactivity was present as bromide ion. None of the injected compound could be detected in urine collected from 2 to 6 hours after injection although small amounts were present in that collected from 0 to 2 hours (Fig. 3).

The high radioactivity found in the stomach and contents (Table I) suggested that here too, the radioactivity might be present as bromide ion, and this was confirmed by paper electrophoresis of the stomach contents of a rat 6 hours after injection of 10 mg. of the compound (Fig. 4).

The rapid liberation of bromide ion from compound (II) *in vivo* suggested that the distribution found may have been typical of injected bromide ion rather than

that of the substituted hydroquinone diphosphate. An experiment to determine the distribution of intravenously injected ammonium ^{82}Br-bromide (0·5 mg.) was therefore carried out on 8 rats bearing a Walker carcinoma. This dose contained the same amount of bromine as 2·5 mg. of 2-methyl-3-bromo-1 : 4-napthohydroquinone diphosphate. The results (Fig. 5) are in agreement with those of other workers (Davenport and Fisher, 1940; Perlman, Morton and Chaikoff, 1941) and show a general similarity to the distribution of radioactivity observed after an injection of the radiobrominated diphosphate. This suggests

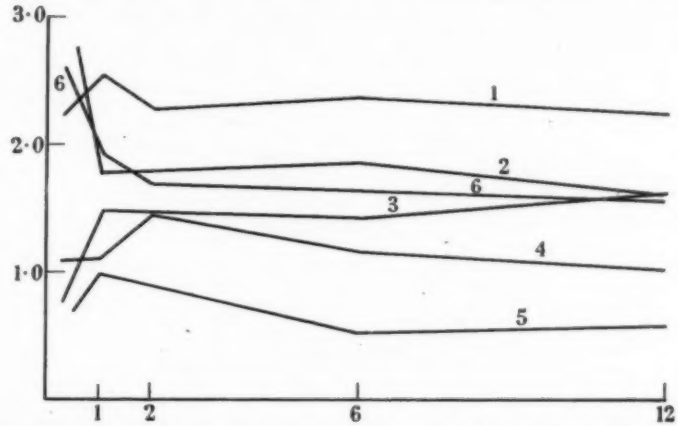


FIG. 2.—Differential absorption ratios of the internal organs of the rat following intravenous injection of 1 mg. (10·6 μC) tetrasodium 2 - methyl - 3 - ^{82}Br - bromo - 1 : 4 - naphtho - hydroquinone diphosphate :

1, tumour; 2, kidney; 3, testis; 4, liver; 5, brain; 6, lung.
Ordinate: Differential absorption rato.
Abscissa: Time in hours after injection.

that the early results shown in Fig. 2 and Table I refer to the distribution of unchanged compound (II), while the later measurements will refer mainly to the distribution of bromide ion.

The facile removal of bromide ion from compound (II) is unlikely to occur by simple exchange since all attempts to affect the reaction *in vitro* were unsuccessful. Although liver and thyroid have been shown to contain enzymes capable of dehalogenating compounds related to thyroxine (Roche, Michel, Michel, Gorbman and Lissitsky, 1953), it is more likely the dehalogenation of compound (II) occurred by dephosphorylation and oxidation to 2-methyl-3-bromo-1 : 4-naphthoquinone followed by reaction with, for example, sulphydryl material (Andrews, Marrian and Maxwell, 1956).

The lability of halogen attached to a hydroquinone ring was also observed with the iodinated compound (III). The D.A.R. of various tissues of a tumour bearing rat at 0·5 and 3 hours after injection of 10 mg. (III) is shown in Table II. The radioactivity of the thyroid is not given separately as it was found to contain no more radioactivity than the surrounding tissue. Again, the D.A.R. of the stomach was so high that dehalogenation was suspected and confirmed by paper

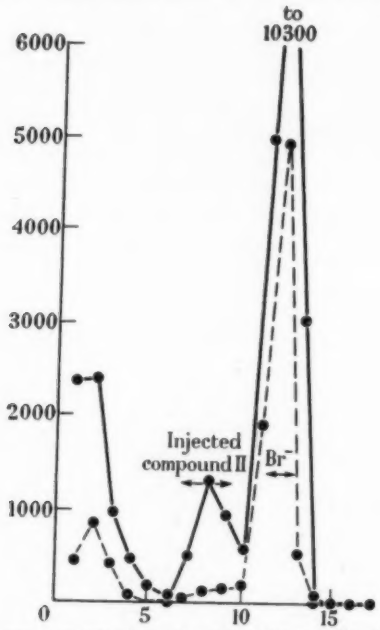


FIG. 3.—Radioactivity along a paper chromatogram of rat urine after injection of 5 mg. of tetrasodium 2 - methyl - 3 - ^{82}Br - bromo - 1 : 4 - naphthohydroquinone diphosphate, showing positions occupied by bromide ion and by the injected compound (II).

Full curve : 0–2 hours.
Broken curve : 2–6 hours.
Ordinate : Radioactivity in counts per minute.
Abscissa : Distance from starting line (arbitrary units).

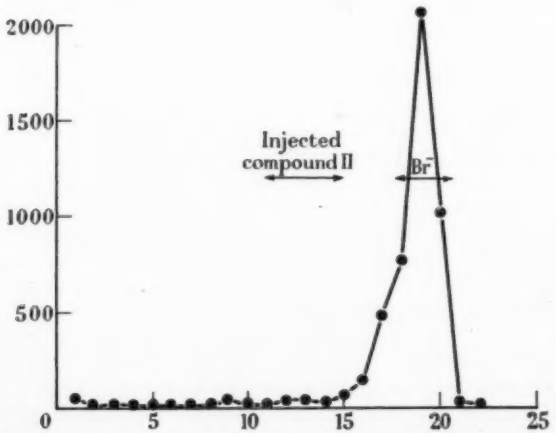


FIG. 4.—Paper electrophoresis of stomach contents of a rat 6 hours after intravenous injection of 10 mg. tetrasodium 2 - methyl - 3 - ^{82}Br - bromo - 1 : 4 - naphthohydroquinone diphosphate showing position occupied by bromide ion.

Ordinate : Radioactivity in counts per minute.
Abscissa : Distance from starting line (arbitrary units).

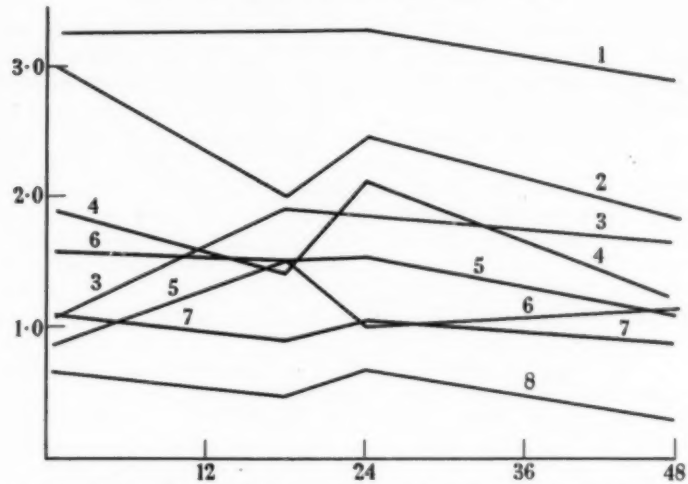


FIG. 5.—Differential absorption ratio of organs of the rat after intravenous injection of 0·5 mg. (10 μC) of ammonium ^{82}Br-bromide.
1, Stomach; 2, lung; 3, tumour; 4, kidney; 5, testis; 6, small intestine; 7, liver; 8, muscle.
Ordinate: Differential absorption ratio.
Abscissa: Time in hours after injection.

TABLE II.—*Differential Absorption Ratios ± Standard Errors*

	0·5 hours	3 hours
Tumour	1·1±0·2	0·7±0·1
Muscle	0·45±0·1	0·3±0·05
Testis	0·55±0·15	0·50±0·05
Liver	0·80±0·05	0·50±0·03
Spleen	0·85±0·05	0·40±0·05
Kidney	2·5±0·5	1·35±0·05
Stomach + contents	8·5±1·6	8·0±0·5
Percentage of Injected Dose		
Blood	11·0±1·0	5·0±0·5
Urine	17·0±3·0	—

electrophoresis both of the stomach contents and of urine collected during the first 3 hours (Fig. 6 and 7).

From these data, we may conclude that the stomach contents and urine of rats receiving the iodo compound (III) contain large amounts of iodide ion and it is of interest to compare the resulting distribution with previous reports of the study of injected iodide. In the mammalian body, the distribution of injected iodide depends on the amount given. A high D.A.R. is shown by thyroid only if very small amounts are administered, and high uptake is then also found in the region of the pituitary (Phillips, 1955). The distrubution of larger doses is said to resemble that of chloride (Wallace and Brodie, 1937), but unlike chloride, the stomach concentrates iodide in the gastric juice relative to plasma (Leblond, 1948; Goldsmith, Stevens and Schiff, 1950). The distribution of 139γ of iodine in sodium ^{131}I-iodide as reported by Stevens, Stewart, Quinlin and Meiken

(1949) is very similar to the distribution shown in Table II, especially at the 0·5 hour period. This confirms that even half an hour after injection, much of the label derived from compound (III) is present as free iodide ion; at this dose

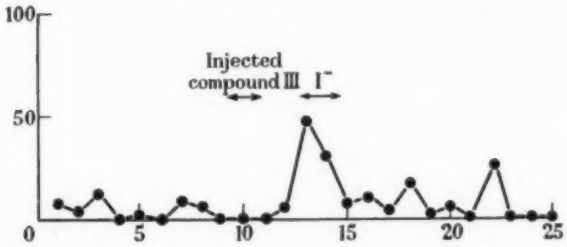


FIG. 6.—The distribution of radioactivity after paper electrophoresis of the contents of stomach of a rat 3 hours after injection of 10 mg. 2 : 3 - dimethyl - 5 : 6 - di^{131}I - iodobenzohydroquinone diphosphate (III) showing the positions occupied by iodide ion and by the injected compound.

Ordinate: Radioactivity in counts per minute.
Abscissa: Distance from starting line (arbitrary units).

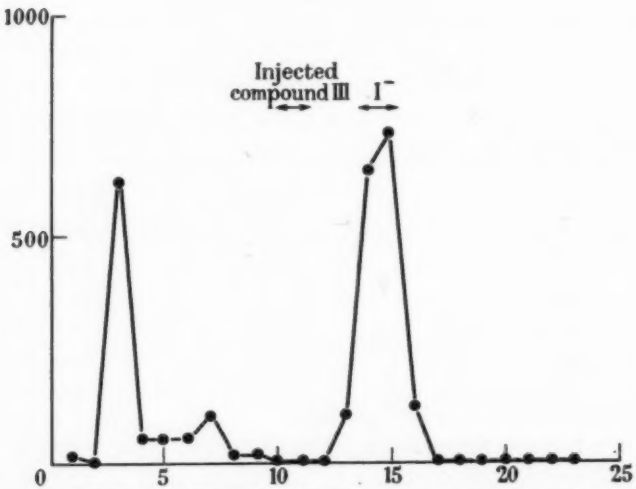


FIG. 7.—The distribution of radioactivity obtained on paper electrophoresis of rat urine collected for 3 hours after injection of 10 mg. 2 : 3 - dimethyl - 5 : 6 - di^{131}I - iodo - 1 : 4 - benzohydroquinone diphosphate (III).

Ordinate: Radioactivity in counts per minute.
Abscissa: Distance from starting line (arbitrary units).

level (10 mg. (III) corresponding to 4 mg. iodine) thyroid would not be expected to have a higher D.A.R. than the surrounding muscle.

The results of these studies indicate the limitations of labelling a potential radiosensitizer of the hydroquinone diphosphate type with halogen in the hydroquinone ring.

SUMMARY

1. The distribution of radioactivity among the organs of rats bearing a Walker carcinoma following injections of 2-methyl-3-^{82}Br-bromo-1 : 4-naphthohydroquinone diphosphate, and of 2 : 3-dimethyl-5 : 6-di^{131}I-iodo-1 : 4-benzohydroquinone diphosphate has been studied.

2. Results show clearly that both compounds accumulate in tumour tissue to a greater extent than in other organs or muscle, but that the halogen atoms are quickly removed from the hydroquinone ring. Therefore, except for short periods after injection, the distribution of radioactivity is that of bromide or iodide ions respectively, with consequent high activity in the stomach contents.

3. The urine of the animals contains large amounts of the respective halide ion.

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SEX DIFFERENCES IN TISSUE CATALASE LEVELS, AND THEIR RELATION TO THE CATALASE-DEPRESSING ACTION OF TUMOURS

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THE pioneer work of Greenstein and his associates showed that a depression in liver catalase activity almost invariably occurs in tumour-bearing rats and mice (Greenstein, Jenrette and White, 1941; Greenstein and Andervont, 1942). The only exceptions were mice bearing extremely slow-growing tumours, and mice of the C57 black strain in which even the rapidly growing Sarcoma 37 produced no significant fall in enzyme activity. However it was found that the liver catalase level in this strain of mice was only about half that found in the other strains used, and the authors suggested that this might be correlated with the insensitivity of its catalase activity to tumour growth (Greenstein and Andervont, 1942).

More recent work, here and in other laboratories, has established the presence in tumour tissue of an agent capable of depressing mouse liver catalase activity *in vivo*. Further, Adams (1950, 1952) found a sex difference in liver catalase activity in the 2 heterogeneous strains of mice used, the male level being higher than the female. On the basis of a series of experiments involving castration, adrenalectomy, and the administration of cortisone and testosterone, it was concluded that the liver catalase activity in these mice is to a large extent maintained by testicular and adrenal hormones. It was then found that the tumour factor affected only the hormonally controlled part of the liver catalase activity (Adams, 1951). If, as these observations suggest, the catalase-depressing action of tumours is an anti-hormone effect, it follows that all strains of rats and mice whose catalase level is affected by tumours should show a sex difference in liver catalase level due to testicular activation, and a decrease in catalase level after adrenalectomy. So far 4 heterogeneous strains of mice examined in this laboratory have all shown a sex difference (males higher than females), and adrenalectomy (performed in 2 strains only) has resulted in a decreased catalase level.

Recently, however, Day, Gabrielson, and Lipkind (1954), who studied liver catalase levels in 6 pure line strains of mice, found a sex difference (males higher than females) in only 1 strain (C58/Bs). In 3 others (C57BL/10Sn, C3H/Fe, C57Br/aSn) the liver catalase levels were approximately equal in both sexes, and in the remaining 2 (AKR/Jax, BALB/c) the female level was higher than the male. These results contrast with those hitherto obtained in this laboratory, and if confirmed would considerably undermine the theory that catalase depression by tumours is an anti-hormone effect, as suggested by Adams (1951). In the present paper, liver catalase levels have been studied in male and female mice of a large number of strains, and in 3 strains of rats; and the opportunity has been taken to estimate kidney and blood catalase levels as well.

According to Greenstein, Andervont, and Thompson (1942) kidney catalase was decreased in the tumour-bearing animal, but to a lesser extent than liver catalase, while blood catalase remained unchanged. Adams and Roe (1953) reported that there was no sex difference in blood catalase levels in their heterogeneous albino strain, but no systematic study of kidney and blood catalase levels appears to have been made. The results to be described show that of the 14 strains of mice tested all have a marked sex difference in kidney catalase, and all but 2 (C57BL and C57Br/cd) show a significant sex difference in liver catalase (males higher than females in both organs). Even in the 2 C57 strains the male liver level was slightly higher than the female.

No sex difference was found in blood catalase levels. The results obtained with 3 strains of rats were similar to the mice.

MATERIALS AND METHODS

Animals.—Young adult mice of the following strains were used. C-, CBA, C57BL, C3Hb, Swiss* kindly provided by the Chester Beatty Research Institute). BALB/c, AKR, A/FA, DBA/1, CE, C57Br/cd Greys* (kindly provided by the Laboratory Animals Bureau, London, N.W.3), Evans Swiss* (commercially obtained), C3H (bred in this laboratory from a strain originally obtained from the Imperial Cancer Research Fund Laboratories. These mice carry the milk factor, in contrast to the C3Hb strain obtained from the Chester Beatty Research Institute, which do not),101, bred in this laboratory from mice originally supplied by Dr. T. C. Carter, Radiobiological Research Unit, Harwell, Didcot, Berks. All the strains are pure lines except those marked * which are colony-bred.

The following pure line strains of young adult rats were also used. J1 albino (bred in this laboratory from a strain originally given to us by Dr. John of St. Thomas' Hospital Medical School), hooded Wistar, and Glaxo albino (kindly provided by the National Institute for Medical Research, Mill Hill).

The diet of the animals consisted of rat cubes (obtained commercially) and water, both *ad lib.*

Estimation of catalase activity.—Liver : the general method has been fully described by Adams, 1950, but in brief, the mice and rats were decapitated and the livers coarsely homogenised in a Ten-Broeck grinder (a glass pestle homogeniser) using ice-cold water. Hitherto the water has been glass distilled, but for the present work " demineralised " water with a conductivity of approximately 5 recip. megohms, obtained from a " Permutit " demineraliser, was used. The resulting suspensions were made up to approximately 10 ml./g. of liver, diluted 10 times with water, and 0·2–0·3 ml. allowed to react with 25 ml. of M/40 hydrogen peroxide in M/50 phosphate buffer pH 6·8 for 4 minutes at 0° C. The reaction was then stopped by the addition of 3 ml. of 50 per cent v/v sulphuric acid, and the hydrogen peroxide remaining estimated by titration with thiosulphate after the addition of excess KI. The catalase activity in arbitrary units was then read from a standard curve. The nitrogen content of the homogenates was determined by Kjeldahl's method and the catalase levels were finally expressed in arbitrary units/mg. N.

Kidney : The same technique was used except that, because of the small amount of tissue available, it was found more convenient to make the original homogenate up to 20 ml./g. and add 0·6 ml. of the final dilution to the hydrogen

peroxide. The standard curve constructed for liver was used, as the relationship between enzyme concentration and hydrogen peroxide decomposed was found to be similar for the two tissues.

Blood : Blood was taken from the cut neck and 0·1 ml. pipetted into 1·9 ml. of water. Of this 0·1 ml. was taken for catalase determination, and 1·0 ml. for N-estimation. Since blood catalase occurs almost exclusively in the erythrocyte, it would have been better to estimate the erythrocyte total N. However, this was impracticable because of the small quantities of blood available, and the N was therefore estimated in whole blood.

Tumour.—Sarcoma 37 (kindly provided in the first instance by the Imperial Cancer Research Fund Laboratories, Mill Hill).

Operative procedure.—Castration was carried out as previously described (Adams, 1952), care being taken to reduce haemorrhage to the minimum.

Hormone.—Testosterone B.P. was obtained commercially.

RESULTS

Table I shows the collected results of catalase estimations on liver, kidney, and blood in mice and rats. The rats were young adults and the mice were about 8–12 weeks old. Males and females of each strain were of the same age, and liver, kidney and blood enzyme levels were estimated in the same mice on the same day.

TABLE I.—*Liver, Kidney and Blood Catalase Levels in Various Strains of Rats and Mice*

The results, in arbitrary units of catalase activity per mg. N, are given as arithmetic means ± standard errors of means. Eight mice and 6 rats per group.

Strain	Liver catalase ♂	Liver catalase ♀	Liver ♂/♀ ratio	Kidney catalase ♂	Kidney catalase ♀	Kidney ♂/♀ ratio	Blood catalase ♂	Blood catalase ♀	Blood ♂/♀ ratio
Mice									
A/FA	241±7·1	183±3·9	1·32	87±6·3	41±3·5	2·12	69±1·0	67±2·1	1·03
AKR	180±4·8	141±5·8	1·28	59±1·8	21±2·8	2·80	65±1·6	74±1·6	0·88
BALB/c	179±6·3	135±5·8	1·33	73±5·1	54±3·2	1·35	65±1·2	66±1·6	0·98
C—	198±3·7	136±6·5	1·45	94±4·9	46±1·8	2·04	47±1·6	49±1·1	0·96
CBA	184±4·0	128±3·0	1·44	74±5·2	42±2·0	1·76	65±3·7	63±1·7	1·03
CE	195±6·3	130±2·9	1·50	75±3·5	36±1·9	2·08	67±0·9	70±2·0	0·96
C3H	236±10·0	155±2·8	1·52	95±8·4	55±4·1	1·73	57±1·1	55±0·6	1·04
C3Hb	200±13·0	135±6·5	1·48	86±1·5	43±2·4	2·00	58±1·4	53±3·0	1·09
,,	188±6·4	113±3·0	1·66						
C57BL	66±1·7	57±1·3	1·15	73±1·8	32±1·4	2·28	32±1·0	32±0·5	1·00
C57Br/cd	72±2·7	69±3·7	1·05	64±3·3	28±1·0	2·28	39±1·0	43±1·6	0·91
DBA/1	197±8·3	120±4·0	1·64	91±7·1	45±1·7	2·02	41±2·1	43±1·5	0·95
101	170±7·1	117±12·8	1·45	104±5·2	39±0·6	2·66	60±2·0	64±2·7	0·98
Chester Beatty Swiss*	174±9·3	111±8·0	1·57	104±9·9	44±3·8	2·45	60±3·6	70±2·2	0·86
Evans Swiss*	156±8·4	117±5·4	1·33	84±5·7	53±2·4	1·58	48±3·1	49±3·2	0·98
Greys*	190±8·6	126±3·2	1·51	76±3·9	38±1·8	2·00	46±4·2	44±2·8	1·04
* Not pure lines.									
Rats									
J1	364±5·2	190±4·4	1·91	122±3·9	73±3·1	1·67	77±2·6	72±4·0	1·07
Hooded Wistar	296±19·0	156±5·6	1·90	146±5·6	79±4·7	1·85	65±2·1	63±4·0	1·03
Glaxo	396±13·3	171±4·6	2·31	168±9·3	95±3·7	1·77	70±3·0	72±2·4	0·97

There are several points of interest in Table I. In mouse liver the only strains showing a negligible sex difference in catalase were the C57 brown and black mice. The levels of activity in these strains were also very much lower than in any of the others. The average ratio of male/female activity in the other strains was just under 1·5 : 1. This agrees very well with the ratios previously found in this laboratory for Glaxo F.F. mice (Adams, 1950), and for Schofield and Schneider albinos (e.g. Adams and Berry, 1956).

The catalase activity in kidney was less than in liver ($\frac{1}{2}$ to $\frac{1}{3}$) but the male/female activity ratio was higher in every strain, averaging just over 2 : 1. The kidneys of C57 mice had catalase activities which did not differ markedly from the other strains, and a large sex difference, in contrast to the values derived from liver determination.

No sex difference was found in blood catalase, the average male/female activity ratio being 0·98 : 1.

In rats there was a similar picture. The liver and kidney catalase activity in all 3 strains was appreciably higher than that of the mice, the blood levels being more or less equal. In all 3 strains however the male/female activity ratio in kidney was less than in liver, a reversal of the findings in mice.

Castration experiments

A group of Evans Swiss male mice was castrated, and a number of subsequent estimations were made of liver, kidney, and blood catalase levels. The results, given in Fig. 1, show that the operation was followed by substantial falls in liver and kidney catalase. The final levels corresponded approximately to the normal female level in this strain of mice. Blood catalase remained unaltered. A group of 6 CBA males was also castrated, and catalase determined after 7 days. As Fig. 1 shows, depressions occurred in liver and kidney catalases, the blood level remaining unchanged.

Testosterone injection

A group of Evans Swiss female mice was injected daily with 100 μg. testosterone dissolved in 0·02 ml. of propylene glycol, and liver, kidney, and blood catalase levels measured from the fourth day. After 7 days the hormone injections were discontinued. The results appear in Fig. 2 and show that there was a rise in catalase level in both liver and kidney, but that while in liver the rise was almost completed by the fourth day, kidney catalase did not rise until the eighth day. The liver enzyme level fell rapidly after the eighth day, while in the kidney the level was maintained, or slightly increased, until the eleventh day (*i.e.* 4 days after the cessation of hormone treatment). Fourteen days after the beginning of the experiment the enzyme in both tissues had returned to the original level. It therefore appears that the reaction of kidney catalase to injected testosterone is very much slower than that of liver. Blood catalase remained unaltered throughout the experiment.

Estimations of liver, kidney, and blood catalase were then made after the injection of tumour homogenate into Evans mice. Both males and females were used. Fig. 3 shows the results of catalase determinations made 1 to 4 days after a single intraperitoneal injection of 80 mg. Sarcoma 37 as a fine homogenate. The liver catalase activity in both sexes was decreased after 1 day, and even more

after 2 days in the males, approximately normal levels being restored by the fourth day. The depression in males was larger than in females. This part of the experiment agrees closely with results already published from this laboratory (Adams, 1950, 1951). The behaviour of kidney catalase was somewhat different. No change in the male level was seen one day after the tumour injection, although at this time the liver catalase had decreased considerably. A depression in male kidney catalase was not obtained until 2 days after the tumour injection, and the

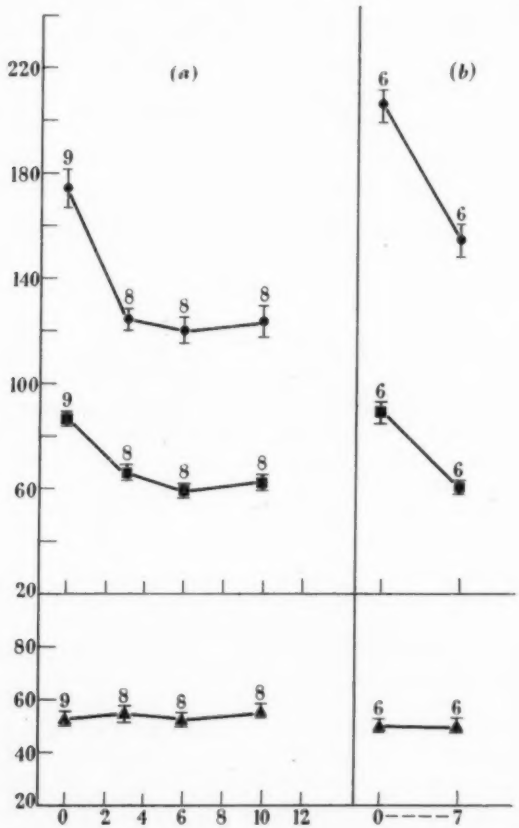

FIG. 1.—Liver, kidney and blood catalase levels in male mice after castration (controls at 0 days). (In this and subsequent figures the results are given as arithmetic means ± standard errors of means. The numbers of animals in the groups are given by the small figures at the head of the standard error limits.)

●——● Liver catalase.
■——■ Kidney catalase.
▲——▲ Blood catalase

(*a*) Evans Swiss mice. (*b*) CBA mice.

Ordinates : Catalase level in arbitrary units/mg. N.
Abscissae : Days.

level subsequently rose slowly to normal. No change could be detected in female kidney catalase, or in the blood enzyme in both sexes. The experiment was repeated using a larger dose of tumour homogenate (100 mg.) which was repeated after 24 hours. Catalase levels were measured 1, 2, and 3 days after the first injection (Fig. 4).

The general pattern of the results resembled those seen after the injection of a single dose of tumour homogenate (Fig. 3), but the male liver and kidney, and

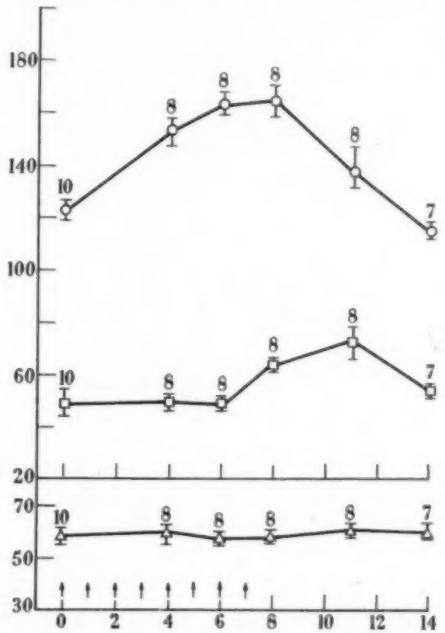

FIG. 2.—Effect of daily injections of 100 μg. testosterone (marked by ↑) on liver, kidney and blood catalase levels of Evans Swiss female mice. (Controls at 0 days.)

○——○ Liver catalase.
□——□ Kidney catalase.
△——△ Blood catalase.

Ordinates : Catalase level in arbitrary units/mg. N.
Abscissae : Days.

female liver, catalase activities were depressed to a greater extent at 2 and 3 days. Again no change in the male kidney level was seen until the second day of the experiment. This time however there also appeared to be a slight decrease in female kidney catalase activity on the second day. Blood catalase remained unchanged.

DISCUSSION

It is disconcerting to find that strongly contrasting results can be reported from different laboratories on what should at first sight be a relatively straight-

forward problem—the evaluation of relative catalase levels in mice. Day, Gabrielson, and Lipkind (1954) reported that while the male liver catalase level in C58/Bs mice was higher than the female, there was no sex difference in C57BL/10Sn, C57Br/aSn, and C3H/Fe mice; and that in BALB/c and AKR/Jax mice the female level exceeded the male. The experiments reported in the present

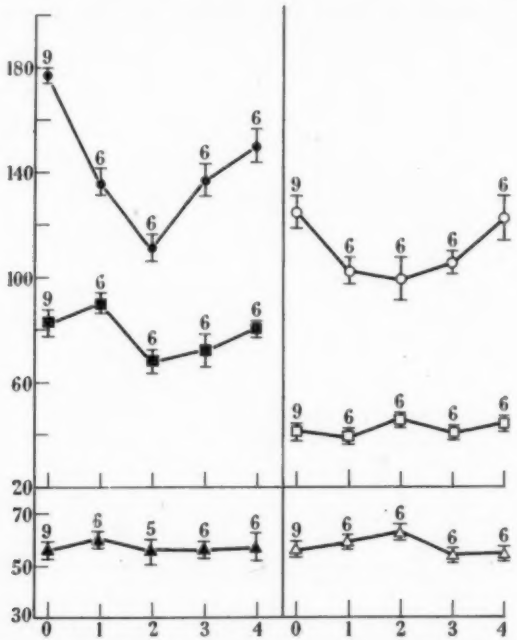

FIG. 3.—Liver, kidney and blood catalase levels in Evans Swiss mice after the injection of 80 mg. S37 (as homogenate). (The controls are at 0 days.)

● ——— ● Male liver catalase.
■ ——— ■ Male kidney catalase.
▲ ——— ▲ Male blood catalase.
○ ——— ○ Female liver catalase.
□ ——— □ Female kidney catalase.
△ ——— △ Female blood catalase.

Ordinates: Catalase levels in arbitrary units/mg. N.
Abscissae: Days.

paper agree in showing only a slight sex difference in liver catalase level in C57BL and C57Br/cd mice; but a marked sex difference (males higher than females) was found in C3H (milk factor) and C3Hb (no milk factor) mice, in BALB/c and AKR mice, and in fact in all the other strains of mice and rats used. These results are in direct contrast with those of Day *et al.* (1954).

One point on which Day, Gabrielson, and Lipkind's (1954) results differ from those of Greenstein and Andervont (1942) concerns the relative catalase level of C57BL mice. The latter authors reported that C57BL mice had a liver catalase

activity approximately half that of the other strains studied, whereas Day *et al.* found the activity of these mice to be high and similar to that of other strains.

They suggested that this discrepancy was due to the fact that they used whole homogenates, while Greenstein and Andervont (1942) used the supernatant from centrifuged homogenates, for the estimation of catalase levels. However, the work reported in this present paper was also done with whole homogenates,

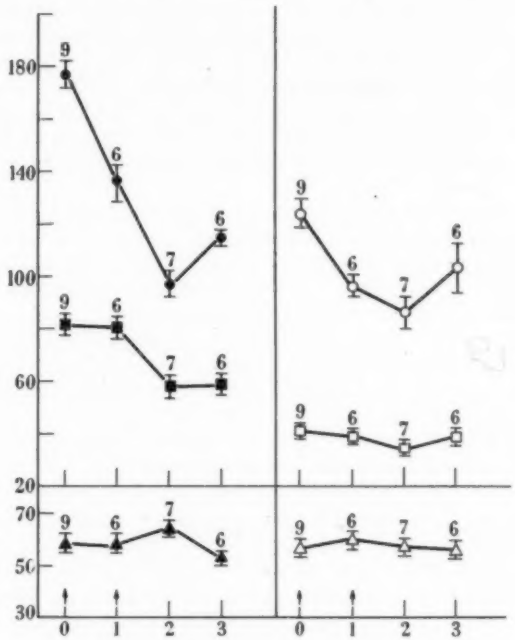

FIG. 4.—As Fig. 3, but two injections of 100 mg. S37 were given at the point marked ↑. The The control groups are the same for Fig. 3 and 4.

Ordinates : Catalase levels in arbitrary units/mg. N.
Abscissae : Days.

yet the liver catalase level in C57BL (and C57Br/cd) mice (Table I) was found to be much lower than in the other strains, in substantial agreement with Greenstein and Andervont (1942). It may be pointed out here that the brown and black C57 strains were derived originally from common parents. C57Br mice were not studied by Greenstein and Andervont (1942).

It seems reasonable to suggest that these discrepancies are due either to differences in the method of catalase assay or in the method of tissue preparation. The task of finding the cause is not made easier by the fact that practically every worker on catalase has his own techniques for both tissue preparation and enzyme assay. It is true however that, as Day *et al.* (1954) have pointed out, very little attention has been given to methods of tissue preparation. Possibly the various

methods of preparation result in the liberation and measurement of catalases in different tissue fractions—some of which are hormonally controlled and tumour sensitive, while some are not. The method of homogenisation used in this laboratory does little more than break up the liver cells—while Day *et al.* (1954) use the much more drastic method of homogenisation in a Waring blender in the presence of octanol-2. Adams and Berry (1956) have recently shown that incubation of liver slices resulted in the liberation of a large amount of additional catalase activity. Since the same extra amount was liberated in both males and females the sex difference activity ratio in catalase activity was considerably reduced. Adams and Berry (1956) also found that the enzyme liberated during liver slice incubation was relatively tumour-insensitive. The results of Day *et al.* (1954) raise the possibility that the additional activity found by Adams and Berry (1956) may be liberated by other methods of tissue treatment—even perhaps by more drastic homogenisation. A study of this problem is being made in this laboratory at the present time.

The results reported in the present paper are consistent with other previous findings. A sex difference in rat liver catalase level (males higher than females) has been reported by Schultz and Kuiken (1941), and by Hargreaves and Deutsch (1952). The failure to influence blood catalase by tumour growth (Greenstein, Andervont, and Thompson, 1942), or by injections of tumour homogenate (Fig. 3 and 4), may be correlated with the absence of a sex difference in blood catalase levels. The insensitivity of liver catalase in C57BL mice to tumour growth (Greenstein and Andervont, 1942) also may be correlated with the low sex difference in this strain (Table I).

Kidney catalase did not react to testosterone until 8 days after the start of injections, while the liver enzyme level rose after 4 days. The injection of tumour homogenate, which produced highly significant depressions in liver catalase activity after 1 day, did not affect kidney catalase until 2 days after injection. It is apparent that testosterone acts more slowly on kidney than on liver catalase, and this may explain the lower sensitivity of kidney to tumour homogenate.

As with liver, male kidney catalase was depressed to a greater extent than female. In fact only a very slight decrease in female kidney catalase was observed even after large doses of tumour homogenate.

No effect of either hormones or tumour homogenate on blood catalase was observed. The finding that catalase sensitivity to tumours is only present in those tissues where hormonal influences can be demonstrated may be taken as additional evidence in support of the hypothesis previously put forward (Adams, 1951) that the change of catalase activity produced by tumours is an anti-hormone effect.

SUMMARY

(1) Liver, kidney, and blood catalase levels have been measured in 14 strains of mice, and in 3 strains of rats. All the male mice had much higher kidney catalase levels than the females, and the males of all strains except 2 (C57BL and C57Br/cd) had higher liver catalase levels than the females. No sex difference was present in blood catalase level. The 3 strains of rats showed a sex difference in liver and kidney catalase levels, but not in blood.

(2) Castration reduced liver and kidney catalase levels in 2 strains of mice examined. Blood catalase was unaffected.

(3) Injection of testosterone into female mice resulted in a rise in catalase level in liver and kidney but not in blood. Kidney catalase took longer than liver catalase to react to testosterone.

(4) Injection of tumour homogenate depressed catalase activity in liver and kidney but not in blood. Liver catalase was affected before kidney catalase. The liver and kidney enzymes in males were affected by tumour homogenate to a greater extent than the corresponding enzymes in females.

My thanks are due to Dr. M. H. Salaman for his interest, to Mr. P. Diamond for skilled technical assistance, and to Mr. J. A. Rawlings for his care of the animals. I am also grateful to the Chester Beatty Research Institute, the National Institute for Medical Research, and the Laboratory Animals Bureau, for their kindness in providing most of the strains of mice and rats used. The expenses of this research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

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THE POLYSACCHARIDES OF A HUMAN CARCINOMA

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THE composition of mesenchymal tumour polysaccharides has been repeatedly investigated: Kabat (1939) obtained from two fowl sarcomas a substance very similar to the hyaluronic acid of human umbilical cords and Pirie (1942) isolated hyaluronic acid from Rous and Fujinami myxosarcomas. Warren and co-workers (1949) reported that the hyaluronic acid extractable from fresh Rous chicken sarcomas had a lower viscosity than the one usually extracted from umbilical cord. Previous data of Sylvén (1945, 1949) and of successive extraction and fractionation experiments (Harris, Malmgren and Sylvén, 1954) suggest the simultaneous presence in the ground substance of mesenchymal tumours of an ester sulphate polysaccharide, which is believed to be a heparin-like one.

In this paper a similar detailed investigation on the polysaccharides extractable from a human carcinoma is reported. Present data will be compared with those for the polysaccharides of Rous sarcoma No. 1 and of human umbilical cords prepared by the same extraction and purification methods.

MATERIAL AND METHODS

The tumour tissue, an undifferentiated carcinoma originating from the pancreas, was obtained from a male patient, aged 66, who died in January 1949. It was supplied by Dr. D. A. G. Galton and Dr. R. J. C. Harris, Chester Beatty Research Institute, London.

The extraction and the purification of polysaccharides from the acetone-defatted and dried material was carried out according to Harris, Malmgren and Sylvén (1954). The extraction schemes are shown overleaf.

The corresponding preparations from umbilical cords were denoted (*U–I*) to (*U–V*).

Each fraction was analysed for nitrogen (Kjeldahl), sulphur (Paulsson, 1953), phosphorus (Lowry and Lopez, 1946) and hexosamine (Blix, 1948) after hydrolysis for 24 hours in 4 M HCl at 100°C. The hexosamine was identified by ion exhcange chromatography combined with automatic recording of electrolytic conductivity according to Drake and Gardell (1952).

Determinations of the elctrophoretic mobility and sedimentation coefficient were carried out at a concentration of 0·5 per cent in phosphate buffers of pH = 7·6 and 6·8 at an ionic strength of 0·1.

RESULTS

The analytical and physico-chemical data are given in Table I.

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TABLE I.—*Analytical and Physico-chemical Data*

	Umbilical cord polysaccharides			Carcinoma polysaccharides			Rous sarcoma No. 1 polysaccharides*	
	(U–I)	(U–II)	(U–V)	(C–I)	(C–II)	(C–V)	(S–I)	(S–II)
Nitrogen in per cent	3·80	3·24	6·20	2·95	3·12	4·20	3·11	3·29
Sulphur in per cent	1·00	0·01	1·03	0·57	0·01	0·47	0·80	0·01
Phosphorus in per cent	0·07	—	—	0·10	—	—	0·05	—
Hexosamine in per cent	30·9	44·4†	25·0	29·8	31·8	29·1	43·0	42·2
Hexosamine in relative amount: Glucosamine	81	100	84	100	100	100	—	100
Hexosamine in relative amount: Galactosamine	19	—	16	—	—	—	—	—
Electrophoretic mobility × 10^5 cm.^{2}/volt sec. at + 0·5° C.	10·0	—	10·7	10·2	—	10·4	9·9	9·9
Sedimentation coefficient Svedberg units	—	1·92	2·35	—	1·01	1·38	1·08	0·98
Optical rotation $[\alpha]_D^{20}$	—	−35° to −40°*		—	−53°	—	—	−44°

* According to Harris, Malmgren and Sylvén (1954).

† Theoretical value; the values obtained are usually about 10 per cent (absolute) lower.

The total yield of polysaccharide material from the human carcinoma was about 2·5 per cent of the fat-free dry weight. Scheme 1 and Table I show that about 90 per cent of this is hyaluronic acid. The chemical composition and the electrophoretic mobility are quite similar to those of purified hyaluronic acid from human umbilical cords and from Rous fowl sarcoma. Infra-red spectroscopy (kindly performed by Dr. S. F. D. Orr) has also shown that the spectrum of preparation (*C–II*) is very similar to that of pure hyaluronate prepared from human umbilical cord and from Rous sarcoma (Orr, Harris and Sylvén, 1952). The two spectra were identical in the region 680–980 cm.$^{-1}$, indicating that the two samples have the same molecular skeleton (Orr, 1954). The sedimentation coefficient of the carcinoma polysaccharide is similar to that of sarcoma polysaccharide; however, it is lower than that of the corresponding umbilical cord preparation (*U–II*).

The sulphur-bearing constituent present in preparation (*C–I*) is completely removed by the Azure A precipitation method of Sylvén and Malmgren (1952) and is accordingly expected to be an ester sulphate polysaccharide. No galactosamine could be detected in (*C–I*) nor in (*S–I*) and the presence of chondroitin-sulphuric acid in measureable amounts, namely more than 2 per cent of total polysaccharide, is therefore excluded. The sulphur-containing substance in (*C–I*) is probably heparin or a sulphate ester of hyaluronic acid. However, none of the preparations labelled (*C–I*), (*C–II*), (*U–I*) and (*U–II*) showed antithrombic activity. This suggests that heparin is absent or that the heparin activity has been destroyed during the preparation.

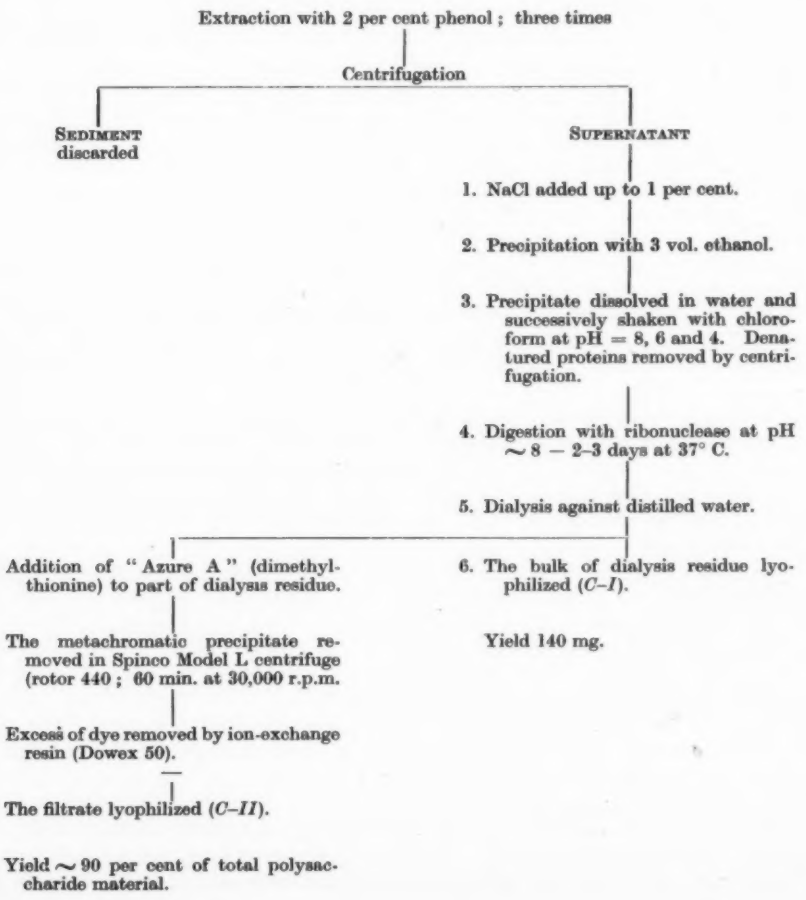
The extraction of the carcinoma with detergents (Snellman) yielded an impure hyaluronic acid (*C–V*), and neither of the fractions (*C–III*), (*C–IV*) or (*C–V*) had antithrombic activity. The first two fractions were obtained in amounts too small to permit chemical analysis. The sedimentation coefficient of (*U–V*) is higher than that of (*C–V*) and the latter contains only glucosamine whereas the umbilical cord preparation (*U–V*) contains both glucosamine and galactosamine.

Extraction of the carcinoma according to Wilander (1938) yielded a sulphur-containing polysaccharide moiety (*S* content 1·04 per cent) that did not, however, exhibit heparin activity. Extraction of umbilical cords gave a similar material.

DISCUSSION

This human undifferentiated carcinoma contained about 2·5 per cent polysaccharide material (fat-free dry weight). About 90 per cent of this was undoubtedly hyaluronate for its chemical properties which were identical with those of hyaluronic acid from human umbilical cord. The carcinoma hyaluronate, however, was of lower particle size than the umbilical. All these findings are in agreement with the observations of Warren and his co-workers (1949) and also of those of Harris, Malmgren and Sylvén (1954) for the hyaluronate of Rous fowl sarcoma,

Scheme 1: Extraction and Purification Scheme of Crude Defatted Tumour Material ((*C*) 5 g.) for Polysaccharides


Extraction with 2 per cent phenol; three times
Centrifugation
SEDIMENT discarded
SUPERNATANT
1. NaCl added up to 1 per cent.
2. Precipitation with 3 vol. ethanol.
3. Precipitate dissolved in water and successively shaken with chloroform at pH = 8, 6 and 4. Denatured proteins removed by centrifugation.
4. Digestion with ribonuclease at pH ~ 8 — 2–3 days at 37° C.
5. Dialysis against distilled water.
Addition of "Azure A" (dimethylthionine) to part of dialysis residue.
The metachromatic precipitate removed in Spinco Model L centrifuge (rotor 440; 60 min. at 30,000 r.p.m.
Excess of dye removed by ion-exchange resin (Dowex 50).
The filtrate lyophilized (*C–II*).
Yield ~ 90 per cent of total polysaccharide material.
6. The bulk of dialysis residue lyophilized (*C–I*).
Yield 140 mg.

indicating that mesenchymal and epithelial tumour hyaluronates are quite similar.

The sulphur-containing polysaccharide which constituted about 10 per cent of the total polysaccharide has not been identified. It contains only glucosamine and cannot therefore be chondroitin-sulphuric acid. On the other hand no antithrombic activity was observed, indicating that heparin or heparin-like substances such as the sulphate esters of hyaluronic acid, were not present in an active

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As preliminary data indicated the presence of a sulphur containing polysaccharide, part of the crude tumour tissue (*C*) was extracted for heparin according to Wilander (1938), and part by a milder method used by O. Snellman (personal communication). The following scheme was adopted :

Scheme 2 : Extraction Scheme of Crude Defatted Tumour Material ((*C*) 3 g.) for Heparin and other Polysaccharides

Extraction in 0·5 per cent Duponol (detergent).
50 per cent saturation with $(NH_4)_2SO_4$.
Centrifugation.
Supernatant dialyzed against water.
Ba-acetate added to 3 per cent (w/v).
PRECIPITATE
SUPERNATANT
Dissolved in Na-salt of ethylene-diamine tetra-acetic acid.
Ethanol added to 20 per cent by vol.
Dyalized and lyophilized.
Yield 4 mg. (*C–III*).
PRECIPITATE
SUPERNATANT
Dissolved in Na-salt of ethylenediamine tetra-acetic acid.
Ethanol added to 50 per cent by vol.
Dialyzed and lyophilized.
PRECIPITATE
Yield 7 mg. (*C–IV*).
Dissolved in Na-salt of ethylene-diamine tetra-acetic acid.
Dialyzed and lyophilized.
Yield 108 mg. (*C–V*).

state. In umbilical cord this sulphur-containing polysaccharide (perhaps a mucoitin-sulphuric acid ?) is not present in appreciable amounts since the sulphur contents of (*U–I*) and (*U–V*) correspond almost exactly with the galactosamine present. The corresponding sulphur bearing polysaccharide of Rous sarcoma also contains only glucosamine, however the sulphur content is higher and an antithrombic activity is detectable (Harris, Malmgren and Sylvén, 1954).

SUMMARY

Extraction experiments show that a human undifferentiated carcinoma, originating from the pancreas, is rich in hyaluronate which has been identified by chemical, spectroscopic and physico-chemical methods. The particle size of this tumour hyaluronate is smaller than that of normal umbilical hyaluronate, but of the same order of Rous fowl sarcoma hyaluronate.

In addition to the hyaluronic acid some unidentified sulphur-bearing polysaccharide was found, which contained glucosamine. The material is provisionally assumed to be chemically related to heparin-like substances, such as sulphate esters of hyaluronic acid, but exhibits no antithrombic activity.

The author wishes to express his sincere gratitude to Professor Arne Tiselius for the privilege of carrying out this work at his Institute, and to Dr. D. A. G. Galton and Dr. R. J. C. Harris of the Chester Beatty Research Institute, for the dried tumour material.

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CHANGES IN VOLUME OF NORMAL ERYTHROCYTES INCUBATED WITH PLASMA OF PATIENTS WITH MALIGNANT TUMOURS

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INTRAVASCULAR haemolysis may occur in malignant neoplastic diseases and is sometimes responsible for anaemia observed in these patients (Stats, Rosenthal and Wasserman, 1947; Hyman, 1954). This observation led us to study the haemolytic activity of plasma of patients with malignant tumours. The preliminary results indicate that although frank haemolysis does not result from incubation of normal erythrocytes with the plasma of these patients, the mean corpuscular volume (MCV) of the incubated red cells is usually increased significantly. No such effect was obtained with plasma of normal individuals, or of patients with benign tumours.

MATERIAL

(1) *Control series*

(a) *Normal controls.*—The plasma of 28 men and women from 25 to 55 years of age was studied. All of these healthy subjects were members of the hospital staff with normal blood count, urinalysis, blood pressure, X-ray of the chest and no gross abnormalities on physical examination.

(b) *Patients with benign tumours.*—This series consisted of 8 patients with the following histologically confirmed benign tumours: Fibro-adenoma of the breast, 1 case; hydatidiform mole, 2 cases; myoma of the uterus, 2 cases; papilloma of the bladder, 1 case; serous cystadenoma of the ovary, 2 cases.

(2) *Patients with malignant tumours*

This series consisted of 15 patients suffering from the following malignant tumours: adenocarcinoma of the breast, 3 cases; granulosa cell carcinoma of the ovary, 2 cases; and 1 case each of: squamous cell carcinoma of the uterine cervix, squamous cell carcinoma of the penis, adenocarcinoma of the endometrium, adenocarcinoma of the ovary, sarcoma of the uterus, serous cystadenocarcinoma of the ovary, papillary transitional carcinoma of the bladder, adenocarcinoma of the colon, generalized carcinomatosis of the peritoneum and mucoid carcinoma of the lung.

METHOD

Blood samples of 10 ml. were taken from the cubital vein and added to 0·05 ml. of a solution of sodium heparin containing 1000 Toronto units per ml. The MCV of erythrocytes was determined from the red cell count and haematocrit reading. The erythrocytes counts were averaged in two Spencer counting chambers.

The haematocrit was determined in Wintrobe tubes centrifuged for 45 minutes at 3000 r.p.m. in an international centrifuge.

The erythrocytes were separated from the plasma in a specially constructed siliconized centrifuge tube with a constricted portion, to facilitate accurate filling to the 4 ml. mark (Fig. 1). After being filled with blood, the tube was centrifuged

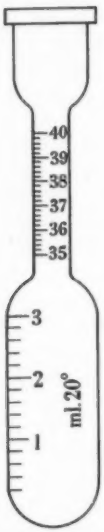

FIG. 1.—Special tube used in incubating blood phases. Overall length 90 mm. Constricted portion allows for accurate filling to 4·0 ml. mark.

at 1500 r.p.m. for 5 minutes. The plasma was then removed and the plasma of another subject of the same blood group added, to bring the total volume again to the 4 ml. mark. The erythrocytes were then resuspended by repeated gentle inversion and the system incubated at 37° C., for one hour. The tube was then again repeatedly inverted and a sample taken for determining the haematocrit. The MCV after incubation was determined by relating this second haematocrit reading to the initial count. Thus repeated erythrocytes counts which might introduce a high degree of error, were obviated.

RESULTS

Neither normal plasma, nor the plasma of patients with benign tumours modified the MCV of normal erythrocytes (Tables I and II). Conversely, normal plasma had no effect on the erythrocytes of patients with benign tumours (Table III).

On the other hand, the plasma of 11 out of 15 patients with malignant tumours significantly increased the MCV of normal erythrocytes (Table IV).

The degree of erythrocytes enlargement did not depend on the extension of the malignant disease, nor the degree of deterioration of the patient's general condition. In fact, the greatest increase in MCV of normal erythrocytes was

produced by the plasma of patients with squamous cell carcinoma of the cervix and squamous cell carcinoma of the penis without evidence of metastases and in fair general condition (Table IV, case 1, 2). On the other hand, no change in the MCV of normal erythrocytes occurred in the plasma of a patient in the terminal stage of adenocarcinoma of the colon (Table IV, case 14).

TABLE I.—*The Effect of Normal Plasma on the Mean Corpuscular Volume (MCV) of Erythrocytes of Other Normal Persons in Incubated Systems*

	Case									
	1	2	3	4	5	6	7	8	9	10
MCV before incubation	78·25	95·18	86·31	85·00	85·71	88·42	86·91	79·79	87·77	90·00
MCV after incubation	78·04	96·40	86·31	85·00	86·73	89·48	85·93	80·80	88·88	90·00
Difference . .	−0·19	+1·22	0·00	0·00	+1·02	+1·06	−0·92	+1·01	+1·11	0·00

	Case									
	11	12	13	14	15	16	17	18	19	20
MCV before incubation	80·00	90·22	78·90	83·87	83·14	89·91	92·68	86·00	85·99	89·37
MCV after incubation	78·94	90·22	81·88	84·95	83·14	88·10	90·24	85·00	88·11	89·37
Difference . .	−1·06	0·00	+2·98	+1·08	0·00	−1·81	−2·44	−1·00	+2·12	0·00

	Case							
	21	22	23	24	25	26	27	28
MCV before incubation	88·42	83·67	91·11	94·30	82·37	80·91	83·51	89·28
MCV after incubation	88·42	82·66	91·11	94·30	82·37	78·84	83·51	88·17
Difference . .	0·00	−1·01	0·00	0·00	0·00	−2·07	0·00	−1·09

Mean of difference was −0·82;
Standard deviation was ±0·84;
Standard error was ±0·16.

TABLE II.—*The Effect of Plasma of Patients with Benign Tumours on the Mean Corpuscular Volume (MCV) of Erythrocytes of Controls in Incubated Systems*

Case	Age	Histological diagnosis	MCV* before incubation	MCV† after incubation	Difference
1	72	Papilloma of the bladder	86·46	88·54	+2·08
2	46	Fibro-adenoma of the breast	82·99	82·99	0·00
3	25	Hydatidiform mole	89·48	89·48	0·00
4	27	" "	85·50	86·00	+0·50
5	53	Myoma of the uterus	91·00	90·00	−1·00
6	49	" " " "	86·04	86·04	0·00
7	53	Serous cystadenoma of the ovary	95·55	95·55	0·00
8	38	" " " " "	80·51	80·51	0·00

*† The difference between the two means was 0, 2;
The standard error was 1·1.
The level of significance (" T Test ") as related to the normal controls series was 0, 2 (not significant).

TABLE III.—*The Effect of Plasma of Control Persons on the Mean Corpuscular Volume (MCV) of Erythrocytes of Patients with Benign Tumours in Incubated Systems*

Case*	MCV before incubation	MCV after incubation	Difference†
1	90·74	87·85	−2·89
2	88·04	88·04	0·00
3	82·66	82·66	0·00
4	90·00	89·00	−1·00
5	89·04	89·04	0·00
6	85·00	85·00	0·00
7	85·50	85·50	0·00
8	92·04	92·04	0·00

* The number of each case corresponds to the case with the same number in Table II.

† Mean of difference was −0·48 ;
Standard deviation was ±0·85 ;
Standard Error was ±0·32.

The level of significance (" T Test ") as related to the normal controls series was 1,2 (not significant).

TABLE IV.—*The Effect of Plasma of Patients with Malignant Tumours on the MCV of Erythrocytes of Normal Persons in Incubated Systems*

Case	Age	Histological diagnosis	Metastases	General condition	MCV before incubation	MCV after incubation	Absolute difference
1	55	Squamous carcinoma of cervix	Absent	Fair	91·13	113·30	+24·63
2	42	Squamous carcinoma of penis	„	„	86·68	101·48	+14·80
3	43	Generalized carcinoma of peritoneum	Present	Poor	85·36	97·56	+12·20
4	37	Adenocarcinoma of the breast	„	Fair	93·00	109·41	+16·41
5	50	Ditto	„	Poor	91·88	97·88	+6·00
6	48	Ditto	„	„	79·00	84·00	+5·00
7	39	Granulosa cell carcinoma of ovary	„	Fair	86·21	93·75	+7·54
8	60	Ditto	„	„	82·35	87·25	+4·90
9	37	Serous papillary cystadenocarcinoma of the ovary	Absent	„	80·93	80·93	0·00
10	60	Adenocarcinoma of the ovary	Present	„	90·24	90·24	0·00
11	66	Mucoid carcinoma of lung	„	„	91·11	97·77	+6·66
12	49	Adenocarcinoma of endometrium	„	Poor	97·43	101·33	+3·90
13	44	Sarcoma of the uterus	„	„	89·74	5·00	+5·26
14	56	Adenocarcinoma of colon	„	„	88·88	88·88	0·00
15	77	Papillary transitional cell carcinoma of bladder	Absent	Fair	86·00	88·00	+2·00

Mean of difference was 7·29 ;
Standard error was ±1·9 ;
Standard deviation was ±6·73.

The level of significance (" T Test ") as related to the normal controls series was 4, 26 (significant.)

When the erythrocytes of patients with malignant disease were incubated with normal plasma, no increase in MCV occurred (Table V).

TABLE V.—*The Effect of Plasma of Control Persons on the Mean Corpuscular Volume (MCV) of Erythrocytes of Patients with Malignant Tumours in Incubated Systems*

Case*	MCV before incubation	MCV after incubation	Difference†
1	93·02	91·08	−1·94
2	90·45	91·71	1·26
3	92·64	94·44	1·80
4	89·48	85·01	−4·47
5	91·88	88·65	−3·23
6	100·00	96·66	−3·34
7	90·44	87·85	−2·59
8	93·54	91·93	−1·61
9	80·00	80·00	0·00
10	92·68	85·36	−7·32
11	85·59	82·35	−3·24
12	88·37	88·37	0·00
13	94·44	94·44	0·00
14	87·30	87·30	0·00
15	89·48	88·42	−1·06

* The number of each case correspond to the case with the same number in Table IV.

† Mean of Difference was −1·64;
Standard deviation was ±2·02;
Standard error was ±0·7.

The level of significance ("T Test") as related to the normal controls series was 1, 1 (not significant.)

DISCUSSION

Increase in the volume of erythrocytes occurs as a first step in the course of the haemolytic process. These prolytic changes in volume develop before complete lysis with hypotonic solutions, saponin and lysolecithin.

The increase in volume of normal erythrocytes on incubation for one hour with the plasma of patients with malignant disease, may also represent a haemolytic phenomenon and suggest that such plasma has increased haemolytic activity.

It has been shown that when normal blood is incubated for prolonged periods of time (12 hours or more), the erythrocytes may undergo prolytic changes in shape with increase in volume (Gillespie, 1943). These changes may be due to the presence of the haemolysins demonstrated in the human plasma incubated at 37° (Bergenhem and Fähraeus, 1936). Incubation is thought to release active haemolysins from inactive complexes with inhibitors (Ponder, 1951). The finding of the haemolytic substances in saline extracts of different tissues suggests possible source of these autohaemolysins.

Furthermore, the haemolytic activity of extracts of malignant tumours is much greater than that of normal tissues (Ponder and Nesmith, 1952). The increase in β glucuronidase and hyaluronidase content of the incubated extracts of malignant tumours may be responsible for their increased haemolytic activity (Leroy and Spurrier, 1955).

It is possible that the property of the plasma of patients with malignant diseases described in this study, may be due to similar enzymatic activity. Our

data appear to indicate that in the conditions we use, the effective concentration of plasma lysins in patients with malignant tumours, is significantly higher than in those with benign tumours or in healthy subjects.

Further work is carried out at present in order to investigate the hypothesis.

SUMMARY

The MCV of normal erythrocytes was significantly increased by incubation with the plasma of 11 out of 15 patients with malignant tumours. The plasma of normal subjects or of patients with benign tumours did not have this effect. It is suggested that the erythrocytes enlargement is prolytic and due to increased haemolytic activity of the plasma of patients with malignant neoplasms.

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DEOXYRIBONUCLEIC ACID IN HUMAN TUMOURS AS MEASURED BY MICROSPECTROPHOTOMETRY OF FEULGEN STAIN: A COMPARISON OF TUMOURS ARISING AT DIFFERENT SITES

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THE malignant cell differs from the non-malignant cell from which it arose in a number of characteristics which, once established, are transmitted from cell generation to cell generation: the ability of the malignant cell to grow, divide and migrate in an uncontrolled fashion, while it may be modified by environmental factors, must have its basis in transmissible characteristics which differ from those of the normal cell. In recent years, the view that deoxyribonucleic acid (DNA) may be responsible for genetic specificity has received increasing support (Avery, MacLeod and McCarty, 1944; Watson and Crick, 1953; Hershey, 1955; Boivin, Vendrely and Vendrely, 1948; Mirsky and Ris, 1949; Brown and Watson, 1953). Although the mechanism of the genetic function of DNA has not yet been elucidated, and there are certain anomalous findings which have still to be confirmed (Chayen and Norris, 1953; Marshak and Marshak, 1955), it is not unreasonable to assume that the amount of DNA in the individual cell nucleus is proportional to the total content of chromosomal material, and thus is probably related to the gene complement of that nucleus. Therefore, while differences in genetic type may or may not exist between cells containing the same quantity of DNA in their nuclei, it is quite likely that cells containing different amounts of DNA have in fact different genetic characteristics.

In this work, we have measured the amount of Feulgen stain in individual cell nuclei of human tumours. According to existing evidence, the amount of Feulgen stain is proportional to the quantity of DNA in cell nuclei. However, it has not been proved that this relationship holds in all cases; for instance where different cell types, having different overall chemical compositions, are concerned, it is possible that the amounts of stain may differ, although the same amounts of DNA are present. It is with this reservation in mind that we use the term DNA content here. The purpose of this study is to investigate the DNA content of human tumour cells and to relate these quantitative data to such information as is routinely obtained in clinical, pathological and cytological studies on the same tumours, as well as to similar data obtained from homologous non-malignant tissues. This first paper is concerned with DNA contents of cells from human tumours arising at various sites.

Before presenting the results it is necessary to consider (a) the reasons for obtaining such data from human tumours, (b) chromosomal variation in the growing neoplasm (i.e. structural and numerical changes in the chromosomes)

and its relation to nuclear DNA content, and (c) the validity of the method we have used for measurement of Feulgen stain.

(a) *The study of human tumours*

In the study of cancer, experimental techniques have been developed which have necessitated the use of transplantable animal tumours. Unfortunately what at first sight would seem to be one of the main advantages of using such tumours, namely that an experiment can be repeated or extended after an interval of time on identical material, does not necessarily hold true, since genetic variation may have occurred in the meantime both in the tumour and in the host strain. Although changes in the former may be eliminated by methods of frozen tumour storage (Craigie, 1954), there remains variation in response to host differences, which may be of great significance even among " strain " animals. Furthermore, and most important of all, each spontaneously-arising human tumour may be genetically unique, and it is thus essential to characterise human tumours by all available means, before data obtained on experimental tumour material may be properly evaluated.

The more immediate objective in studying human tumours has, in most work, been the practical one of providing information of use to the clinician : for instance histological evidence of the probable rate and mode of spread of the tumour, and its probably response to radiotherapy or other forms of treatment. Classical histology has enabled human tumours to be characterised with respect to such features as their degree of differentiation, but little progress has yet been made in the application of cytochemical techniques whereby one may characterise different tumours with respect to their fundamental chemical components. Before any new technique may be applied with confidence to highly variable human tumours, however, much preliminary investigation is essential.

(b) *Chromosomal variation and DNA content*

Genetic variation in tumours may take the form of gene mutations, structural alterations in chromosomes, and changes in chromosome number involving the gain or loss of whole chromosomes. The first of these is not amenable to study at the present time, but microscopical examination can provide information concerning changes in chromosome number and morphology. Thus it has been shown in animal tumours that there are wide differences in chromosome number and type from the chromosome idiogram of the host germ-line (Makino and Kano, 1953 ; Levan and Hauschka, 1953). It must be borne in mind, however, that the tumour chromosomes should be compared only with those of the corresponding homologous normal somatic cells, since, in the latter also, chromosomal variation is believed to exist (Beatty, 1954). This is difficult with transplantable tumours. Little information is available about the chromosomes of human tumours, largely owing to the difficulties experienced in the use of the conventional methods of chromosome study on this material, often coupled with a scarcity of cells in mitosis.

Chromosome studies on animal tumours have, however, revealed several important features of tumour growth. Firstly, abnormal cell division, occurring during the growth of the tumour, may produce variation in chromosome number, since it results in the unequal partitioning of the chromosomal complement between the anaphase groups. The daughter cells may thus have lost or gained whole

chromosomes, or structural changes may have resulted in the appearance of " new " chromosomes. Irregular mitosis occurs in normal as well as malignant tissues, but may be much more frequent in the latter. Another feature of normal cellular growth which may be exaggerated in tumours is the production of polyploid cells. These may arise as a result of abnormal mitosis (Fell and Hughes, 1949), or doubling of the chromosome complement by endomitosis or endoreduplication (Levan and Hauschka, 1953). The presence of similar mitotic irregularities in human tumours has been described by Koller (1947).

Secondly, and probably of far greater importance, repeated sampling of animal tumours has demonstrated the existence of stem-lines : in spite of the apparent inhomogeneity of tumour cell populations, evidence derived from the distribution of chromosome numbers has indicated that the growth of each tumour is nevertheless due mainly to the multiplication of cells bearing a particular chromosome complement, which constitute the stem-line of the tumour (Makino and Kano, 1953 ; Levan and Hauschka, 1953 ; Sachs and Gallily, 1955). The stem-line frequently differs in its chromosome number from the diploid value for the species ; in addition it may bear chromosomes which are distinguishable on morphological grounds from those of the normal diploid set.

Since comprehensive studies of the chromosomes in an unselected series of human neoplasms present very great practical difficulties, an alternative approach which will give information relating to the chromosomal characteristics of each tumour is desirable. This is possible by the method of DNA estimation which, moreover, can be applied to cells in interphase, as well as to cells in all stages of division (Richards, Walker and Deeley, 1956). Since changes in chromosome number may be accompanied by structural changes resulting in a redistribution of chromosomal material, it is possible for two cells to have different chromosome numbers but the same DNA content ; apart from possible gene " position effects " these cells may differ but little in genetical characteristics. The DNA content of a cell, since it does not take into account morphological differences in chromosomes, may bear a more direct relationship to the genotype of the cell than does chromosome number.

(c) *The method of measurement of Feulgen stain.*

Despite earlier criticism of its technical and chemical features (Glick, Engström and Malmström, 1951), the spectrophotometric estimation of Feulgen stain in individual cell nuclei, originated by Pollister and Ris (1947), has been widely employed in the measurement of nuclear DNA. Its validity has been established by the excellent agreement obtained between the results and those of other methods, and by its reproducibility on known material (Leuchtenberger, 1954). Nevertheless, like most quantitative cytochemical methods (though perhaps less than many), it is fraught with technical pitfalls. These must be constantly borne in mind, especially when the significance of small differences in stain content is to be evaluated (Swift, 1953).

Apart from the possible failure of stain specificity and stoichiometry, the measured amount of Feulgen stain may vary owing to inaccuracies in the optical measurement. These have been discussed many times elsewhere (e.g. Davies and Walker, 1953 ; Deeley, Richards, Walker and Davies, 1954), but they are, we believe, smaller in the instrument which we have used (Deeley, 1955) than in those commonly employed elsewhere.

MATERIALS AND METHODS

Small pieces of tissue were placed in ice-cold Earle's solution, immediately after removal at operation or by biopsy. In most cases, about an hour elapsed before fixation, which was done at King's College. Smears were then made on coverslips, and since it was desirable to have a high degree of cell separation, dissociation of the tissue was facilitated by tapping the fresh material with a flat-ended glass rod. The ease of cell separation varied widely with different tumours, being relatively easy in the more anaplastic ones.

The smear of isolated cells and cell nuclei which resulted was immediately fixed by methanol freezing substitution (Simpson, 1941). When necessary, the material was stored in methanol at 2° C, and staining was done shortly before measurement. Feulgen staining was carried out according to the method of Stowell (1947). After removal from methanol, the coverslips were hydrated in the alcohol series, hydrolysed for 7 minutes in N.HCl at 60°C, left in stain for 1 hour, washed in SO_2 water and finally mounted in glycerol from distilled water. Since it was necessary to use the cell-crushing procedure (see below), the coverslips were mounted as described elsewhere (Davies, Wilkins and Boddy, 1954).

The amount of stain in individual cell nuclei was measured by the rapid scanning photoelectric instrument designed and built by Dr. E. M. Deeley (1955) To avoid selection of cell nuclei for measurement, the specimen was scanned systematically, and all those which came within the field stop area were crushed and measured, except occasional overlapping nuclei. In each specimen, between 60 and 100 cells were measured ; a sample of 15–25 polymorphonuclear leucocytes were also measured, together with a sample of fibroblasts or other normal cell types, if these were identified.

Results were plotted in the form of frequency histograms. In each histogram, broken vertical lines denote the control value, and multiples of this value, for polymorphonuclear leucocytes (l, $2l$, etc., where l = mean DNA content of polymorphs). To facilitate comparison of different histograms, the number of class intervals was standardised. Thus near-diploid tumours were plotted with 10 or 11 classes between the l and $2l$ levels, and near-tetraploid tumours with 10 or 11 classes between the $2l$ and $4l$ levels.

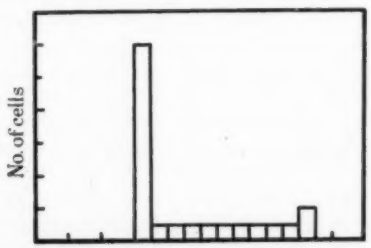

FIG. 1.—Idealised histogram of DNA values in a sample of a population of dividing cells, showing high primary mode, lower secondary mode, and intermediates. The abscissa refers to amount of DNA.

Fig. 1 illustrates the typical form of a histogram of DNA values for a dividing cell population. The primary mode is composed of cells which have the basic quantity of DNA associated with a set of post-telephase diploid chromosomes.

A low secondary mode at double the value of the primary mode is usually apparent, and is composed of (i) " resting " cells which have double the chromosome complement of the main population, and (ii) pre-prophase cells which have completed DNA synthesis. Cells which are in the process of synthesising DNA occupy an intermediate position between these two modes. If aneuploid cells are present, they may be expected to bear non-modal DNA values.

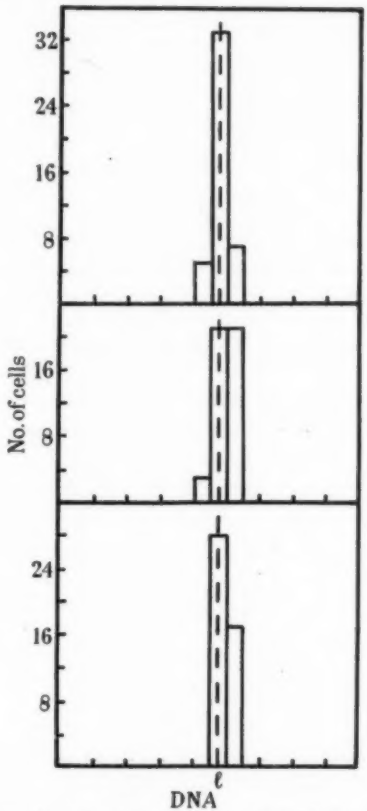

FIG. 2.—Normal cells of mesodermal origin. Polymorphonuclear leucocytes (top), lymphocytes and plasma cells (middle), and fibroblasts (bottom), from a specimen of carcinoma of the cervix.

RESULTS

Normal cells of mesodermal origin

Three categories of normal mesodermal cells were measured. Fig. 2 illustrates the distributions obtained for polymorphonuclear leucocytes, small and large lymphocytes and plasma cells, and tissue fibroblasts respectively. All these results were obtained from measurements made on a single coverslip preparation of tumour material (carcinoma of the cervix uteri). The vertical broken line

corresponds to the mean value of the polymorph distribution, which is 137, S.D. $\pm$ 7·45 arbitrary units. The mean of the lymphocytes, etc. is 141, S.D. $\pm$ 6·36, and that of the fibroblasts 142, S.D. $\pm$ 4·76. All three distributions show a relatively small scatter, suggesting a high degree of constancy of DNA in these normal cell types.

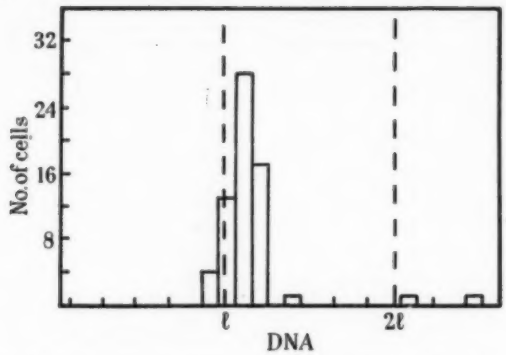

FIG. 3.—Epithelium from apparently normal cervix (patient aged 41, who had undergone hysterectomy for a benign condition).

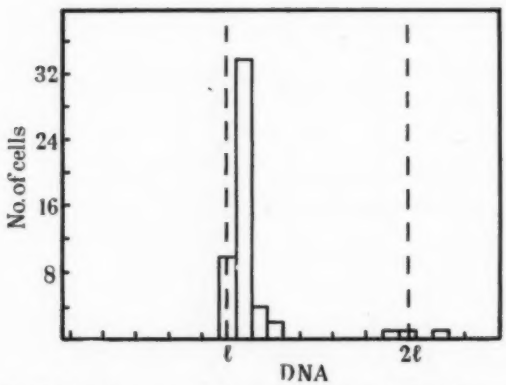

FIG. 4.—Epithelium from non-malignant cervix (cervical erosion; aged 35).

Non-malignant epithelial tissues

Since most of the tumours studied were epithelial in origin, a few specimens of non-malignant epithelial cells from endometrium and cervix uteri were examined. Fig. 3 and 4 show distributions of DNA content in two specimens from the cervix uteri. In both, a major mode is present at approximately 10 per cent above the l value, and a few cell nuclei contain twice this amount. It would appear that a few cells containing tetraploid amounts of DNA may be present in normal cervix uteri. Essentially similar distributions were obtained in two specimens of endo-

metrium (Fig. 5 and 6). In these, however, a few intermediate values are present, which are probably due to cells synthesising DNA prior to mitosis.

The mean DNA values of cells in the modal range in all four histograms of non-malignant epithelial cells are approximately 10 per cent above the corresponding l value. In all the distributions there is only a small degree of scatter about the mode.

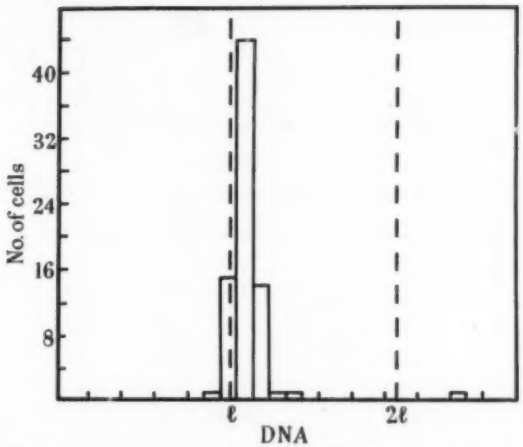

FIG. 5.—Endometrium from same patient as Fig. 4 (27th day of menstrual cycle ; histology : a hyperplastic endometrium which has undergone advanced secretory changes).

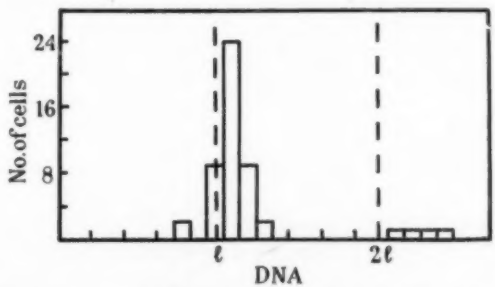

FIG. 6.—Endometrium (non-malignant) from patient aged 41 ; 17th day of cycle.

Tumours

With the exception of those of carcinoma of the breast, all results are from untreated cases.

(i) *Carcinoma of the cervix uteri.*—Three typical distributions of DNA content of cervical carcinoma cells are illustrated in Fig. 7 to 9. Case FY has a well-defined primary mode at 10 per cent above the l value, in contrast to Cases FX and EE which both have primary modes lying at the $2l$ level. Secondary modes

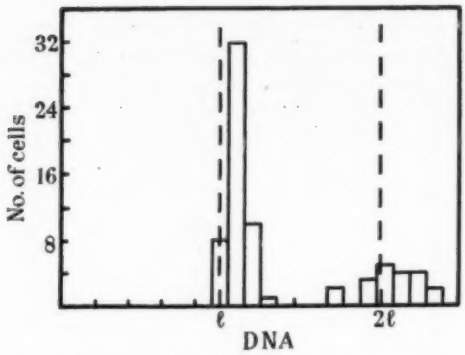

FIG. 7.—Case FY, aged 48. Carcinoma of the cervix, Stage IV. Histology: poorly-differentiated squamous cell carcinoma.

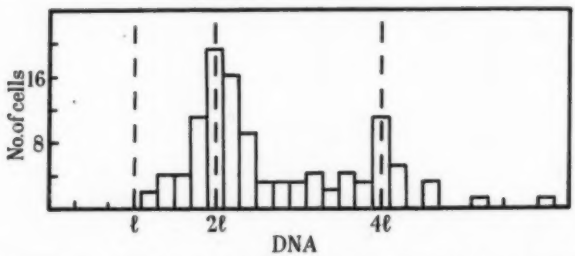

FIG. 8.—Case FX, aged 79. Carcinoma of the cervix, Stage I. Histology: poorly-differentiated squamous cell carcinoma.

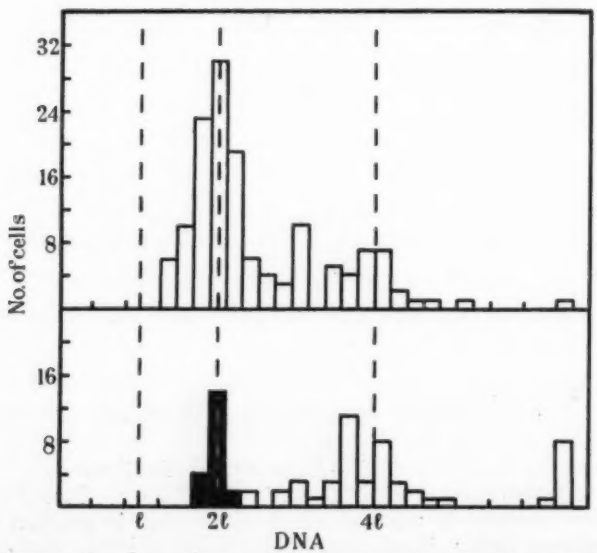

FIG. 9.—Case EE, aged 50. Carcinoma of the cervix, Stage IV. Histology: squamous carcinoma of moderate differentiation and some attempt at keratinisation. Top: interphase. Bottom: open-metaphase and prophase; black—telophase.

and intermediate DNA values, are present in these specimens, but the proportion of cells having intermediate values is far greater in FX and EE than in FY. Specimens from Case EE contained many division stages, and samples of these are also shown in Fig. 9. The DNA contents of prophases and metaphases show a large scatter (mean : 171, S.D. $\pm$ 25·7) in contrast to that of the telophases (mean : 94, S.D. $\pm$ 7). This picture of DNA content of division stages is similar to that previously found in certain animal tumours (Richards, 1955).

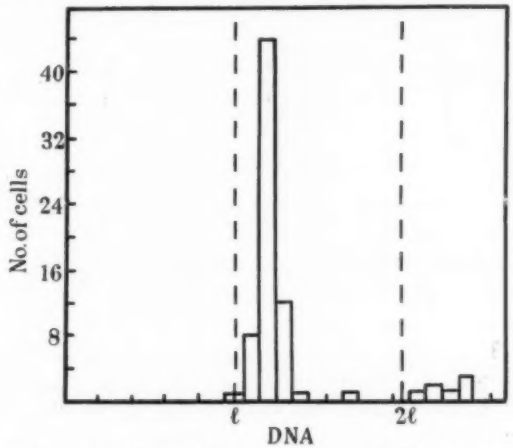

FIG. 10.—Case IR, aged 66. Carcinoma of the corpus uteri. Histology : fairly well-differentiated mucus-secreting papillary adenocarcinoma with foci of squamous metaplasia.

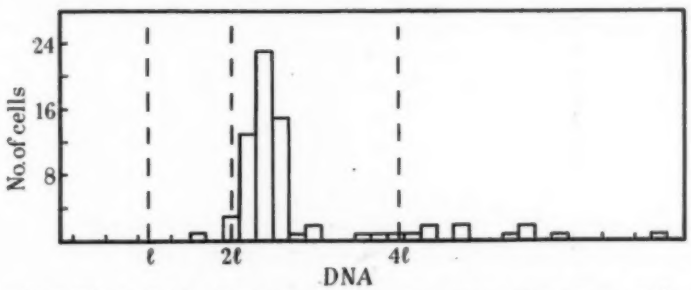

FIG. 11.—Case HC, aged 79. Carcinoma of the corpus uteri. Histology : rapidly-growing fairly well-differentiated columnar cell adenocarcinoma.

(ii) *Carcinoma of the corpus uteri.*—Fig. 10 to 12 show frequency histograms of DNA content in three cases of carcinoma of the body of the uterus. The first (Case IR) shows a prominent primary mode at approximately 20 per cent above the l value. A few intermediates and a small secondary mode are present. Similarly, in Case HC only a few cells contain intermediate amounts of DNA, but here the primary mode is about 20 per cent above the $2l$ value, and there are a relatively large number of intermediate values.

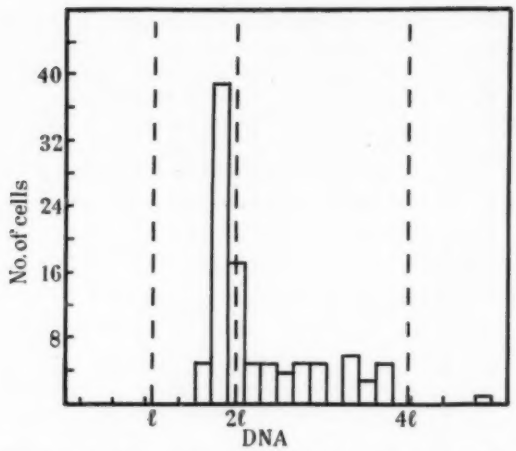

FIG. 12.—Case HE, aged 67. Carcinoma of the corpus uteri. Histology: fairly well-differentiated papillary adenocarcinoma.

(iii) *Carcinoma of the breast.*—Of the two cases illustrated, the first (Case FD, Fig. 13) shows a primary modal DNA content at approximately 25 per cent above the *l* value, while in the second case (EQ) it lies at the 2*l* level. In the latter case, the two histograms represent samples obtained from cutaneous secondaries

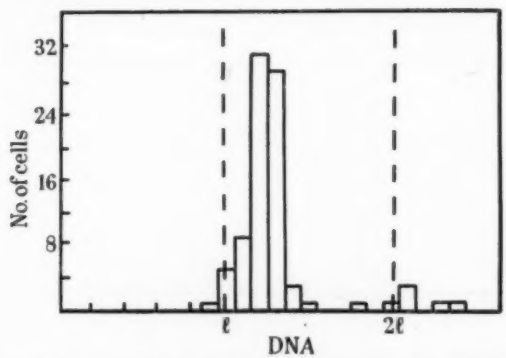

FIG. 13.—Case FD. Female, aged 54. Cutaneous recurrence of carcinoma of the breast. Previously treated by simple mastectomy and post-operative DXR. Histology: spheroidal cell carcinoma.

before and after adrenalectomy (Fig. 14A and 14B respectively). Clinically, the tumour showed no response to adrenalectomy, and no significant difference is discernable in the DNA distributions of the two specimens. In both specimens, a few cells were present which had a DNA content equal to the *l* value. Though not distinguishable from tumour cells, they may have been normal fibroblasts.

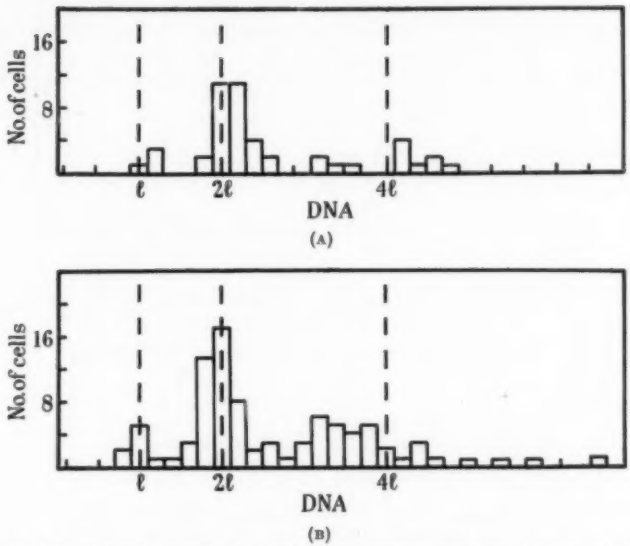

FIG. 14.—Case EQ. Female, aged 53. Cutaneous recurrence of carcinoma of the breast. Previously treated by radical mastectomy and post-operative DXR. Histology: undifferentiated spheroidal cell carcinoma. (A): before adrenalectomy. (B): 5 weeks after adrenalectomy.

(iv) *Squamous cell carcinoma at miscellaneous sites.*—Four cases of squamous cell carcinoma from different sites are shown in Fig. 15 to 18. Primary modes in the carcinoma of the anus and carcinoma of the vagina are 15 and 30 per cent above the *l* value respectively, while in the third and fourth cases (carcinoma of the mucous surface of the cheek, and of the tongue) this mode lies at the 2*l*

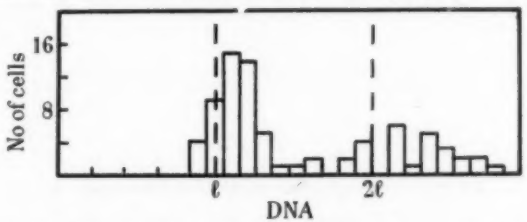

FIG. 15.—Case HU. Female, aged 56. Carcinoma of the anus. Histology: poorly-differentiated squamous cell carcinoma.

level. Intermediate values showing DNA synthesis are present in all four cases. The specimen of carcinoma of the tongue contained many non-malignant cells, including epithelial cells and fibroblasts; the histograms of DNA content for these cell types are in general agreement with those described above for other normal cells (the mean DNA value of the polymorphs is 85 arbitrary units; of the fibroblasts, 87 arbitrary units; and of the non-malignant epithelial cells, 97 arbitrary units).

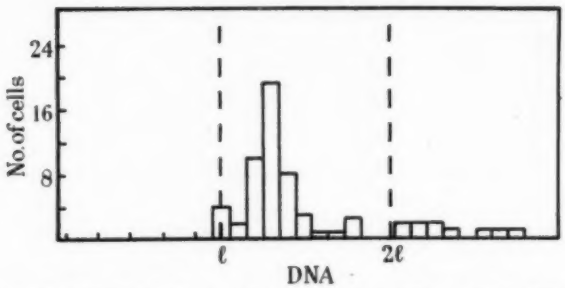

FIG. 16.—Case HX. Female, aged 81. Carcinoma of the vagina. Histology: keratinising squamous cell carcinoma.

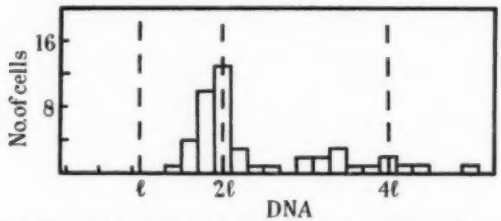

FIG. 17.—Case EG. Male, aged 79. Carcinoma of mucous surface of the cheek. Histology: anaplastic squamous cell carcinoma.

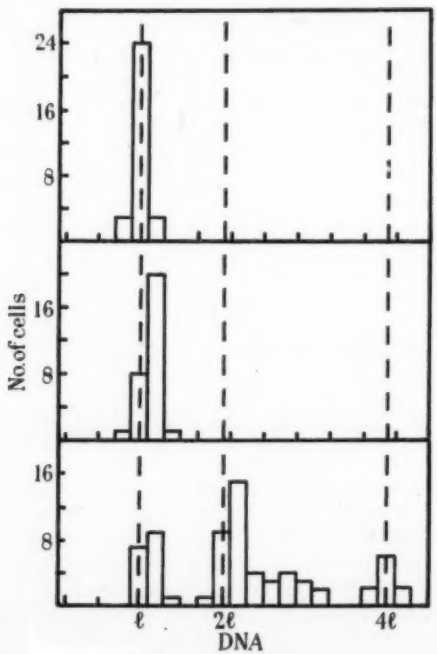

FIG. 18.—Case KX. Male, aged 30. Carcinoma of the tongue. Histology: keratinising squamous cell carcinoma. Top: fibroblasts. Middle: non-malignant epithelial cells. Bottom: tumour cells.

(v) *Other tumours.*—A malignant melanoma (Fig. 19) shows well-defined primary and secondary modes, but a noticeably small number of intermediate values, although this was a rapidly growing tumour. The primary mode is seen

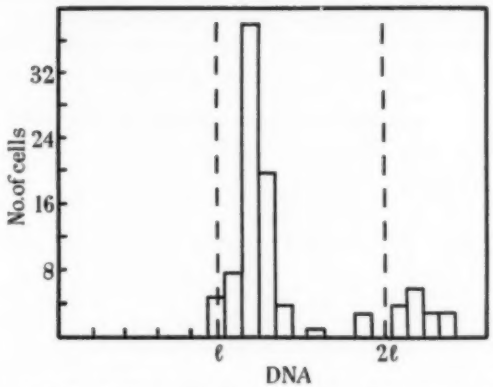

FIG. 19.—Case GG, aged 76. Malignant melanoma of the vulva. Histology: diffuse mass of large polyhedral cells with scanty pigmentation and frequent mitoses.

to lie at just over 20 per cent above the *l* value. A higher primary mode, at 30 per cent above the *l* value, is shown by a leiomyosarcoma of the uterus (Fig. 20), which also has a well-marked secondary mode at twice the value of the primary mode. In the remaining three cases the main modes are in the neighbourhood

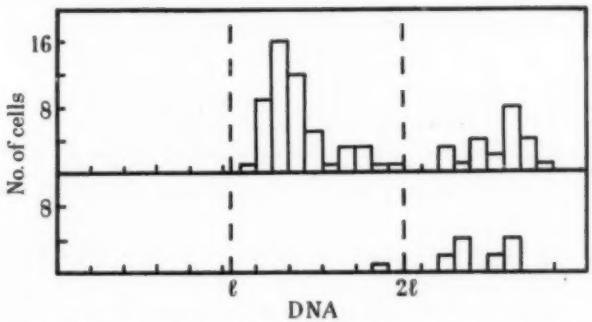

FIG. 20.—Case HM, aged 56. Leiomyosarcoma of the uterus. Top: interphase. Bottom: metaphase.

of the 2*l* value (carcinoma of the stomach, Fig. 21; basal cell carcinoma, Fig. 22; and carcinoma of the rectum, Fig. 23).

DISCUSSION

For each tumour there is a modal DNA value which may be taken to represent the stem-line of the tumour. The stem-line concept has been developed from observa-

tions on animal tumours transmissible in the "ascites" form, and, as already mentioned, carries the implication that the growth of the tumour is mainly due to the multiplication of cells having chromosome numbers at or near the modal value, while cells with numbers deviating widely from this value are likely to be inviable (Makino and Kano, 1953; Sachs and Gallily, 1955). It is considered justifiable to equate the primary mode of the DNA histogram with the chromosome complement of the stem-line cell, because, as pointed out previously, this mode

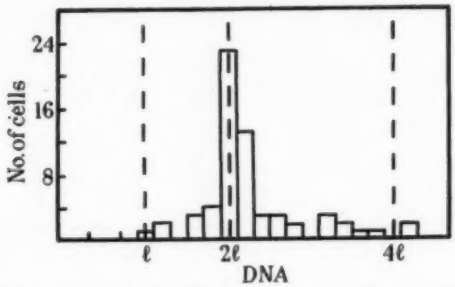

FIG. 21.—Case LI. Female, aged 58. Carcinoma of the stomach (cardiac end). Histology: adeno- and spheroidal cell carcinoma.

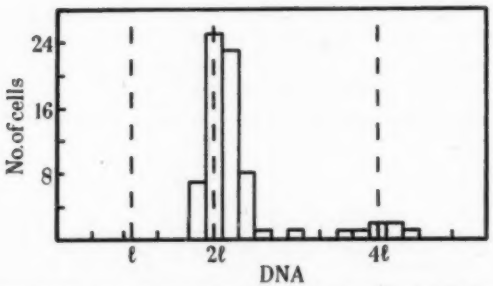

FIG. 22.—Case KZ. Female, aged 76. Basal cell carcinoma of the forehead.

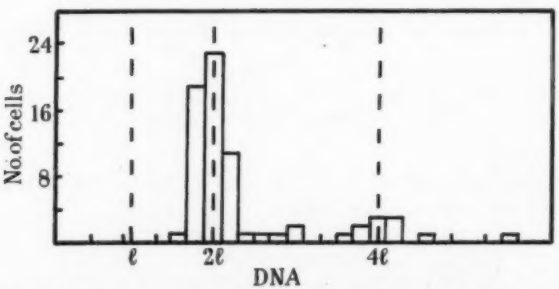

FIG. 23.—Case KC. Male, aged 74. Carcinoma of the rectum. Histology: well-differentiated columnar cell carcinoma.

represents the basic or post-telophase DNA value of the majority of the cells comprising the population. Confirmatory evidence of the position of this mode is provided by (i) a small number of interphase cells with doubled amounts of DNA forming a secondary mode at twice the value of the primary one, and (2) the fact that cells in mitosis also show values which tend to be grouped around this secondary mode, which would accord with the current theory of DNA constancy.

In the present series of observations, it will be seen that the primary modes, taken as a whole, show a tendency to fall into two groups : either about 10–30 per cent above the l level, or near the $2l$ level (Fig. 24). In a further series of

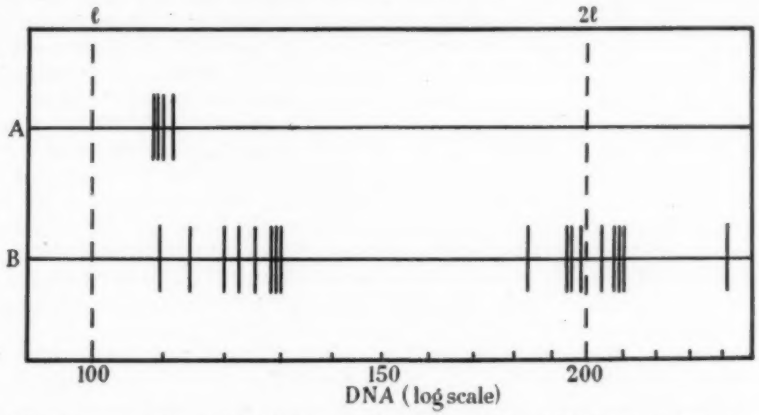

FIG. 24.—Summary of numerical data on basic DNA contents of (A) 4 non-malignant and (B) 17 malignant human tissues arising at various sites. Values are in arbitrary units, with reference to polymorphonuclear leucocyte value taken as 100.

observations on carcinoma of the cervix, which will be discussed in a subsequent paper, there is a similar tendency ; occasionally, however, the mode falls wide of these two levels. It seems that two processes may have occurred to produce the difference between the DNA content of the modal (stem-line) cancer cell and the non-malignant cell : firstly, a fairly small change in DNA content (*circa* 10 per cent), which in the lower group of the present series is always in an upwards direction, and, secondly, in some cases (those forming the upper group), there may be a doubling of the chromosome complement. While the near-diploid tumours, however, have DNA modes in the region of 10–30 per cent above the leucocyte level, the higher or " tetraploid " group in general have modes which are not similarly raised above twice the leucocyte value, but are grouped around this value. From the observed differences between non-malignant epithelial cells and leucocytes, it would seem that tumours falling into the latter group are hypotetraploid as regards DNA content, and thus resemble the " tetraploid " experimental tumours of mice, which frequently have a hypotetraploid number of chromosomes (Levan, 1956).

The results show that " tetraploid " tumours are common among human neoplasms, as they are among experimental animal tumours. In the latter, repeated observations over many transplant generations have revealed that

individual tumours may change from near-diploid to near-tetraploid. The change in these animal tumours has been correlated with a loss of host-specificity, since " tetraploid " tumours can on the whole tolerate a greater range of host genotypes than can diploid (Hauschka and Levan, 1953). When a near-diploid mouse tumour is transplanted into a host whose genotype differs from that of the original host, a selective mechanism is brought into play which may favour the development of a new-stem-line derived from cells with a higher chromosome number. Such a selective mechanism is of course absent in human neoplasms, and whether or not the " tetraploid " tumours arose from tetraploid cells already present in the tissue of origin is not known.

We shall briefly consider how the results described above compare with those obtained by other workers on the DNA content of tumours. Data obtained by biochemical techniques can only be expressed in terms of average amount of DNA per cell, and, although they may indicate whether a tumour is approximately diploid or tetraploid, give little indication of the true position of the basic mode, and may give misleading results where many non-tumour cells are present. DNA measurements on animal tumours have been made by various authors, and in general have agreed fairly well with chromosome counts. Leuchtenberger and her collaborators (Leuchtenberger, Leuchtenberger and Davis, 1954) have measured a number of normal and malignant human tissues by a microspectrophotometric technique. They found that the " mean basic DNA content " of the normal tissues varied little, that in 14 tumours the basic DNA content was the same or only slightly higher than this value, in 8 tumours it was approximately 30 per cent higher, and in 7 the lowest modal value of the cells was " tetraploid ".

As compared with other workers, we have perhaps laid stress on smaller differences in DNA content (e.g. the 10 per cent difference we have found between non-malignant epithelial and mesodermal cells) than might elsewhere be considered significant. We think, however, that our technique permits considerable accuracy of measurement; moreover, we have not been concerned with the measurement of nuclear areas, as required for some methods, nor, since we have used smears instead of sections, with the presence of incomplete nuclei. The small difference between the modal value of the non-malignant epithelial cells on the one hand and the leucocytes and fibroblasts on the other, has been repeatedly found and is considered significant. Since, as mentioned in the introduction, nuclei of different types are concerned, these differences in the amount of stain measured may not necessarily represent true differences in DNA content. The constancy of the ratio between the modal values of the non-malignant epithelial and mesodermal cells, however, seems to justify our comparing samples of different tissues by the indirect method of relating the values obtained for each tissue to those of the leucocytes present in that tissue; furthermore, since we have found slightly higher values for non-malignant epithelial tissues than for leucocytes, the diploid DNA value of the homologous normal tissue for epithelial tumours (at least for those of uterine origin) would appear to be 10 per cent above the mean leucocyte value.

It is of interest that the basic DNA content of the tumours that we have examined in most cases shows a difference from the normal. It has frequently been suggested that the malignant state represents a reversion to a primitive type, involving the loss of specialised functions and simplification of nutritional requirements; thus, it may be argued that the number of essential " gene-

enzyme " complexes or genes necessary for the growth of the malignant cell is less than that of normal specialised cells. Hence, although it does not necessarily follow, it might have been expected that the DNA content would be less in tumour cells than in normal cells. However, we have in most cases found an increase in the DNA content of the malignant cell in comparison with the normal. In this connexion, it has been suggested by the work of Fautrez and his group (Fautrez, Pisi and Cavalli, 1955 ; Fautrez, Cavalli and Pisi, 1955) that in normal tissues there may be an increase in the basic DNA content which is associated with cell division. We have not always found increased DNA content in human tumour cells, for example Case FY, Fig. 7 (carcinoma of the cervix), which showed a DNA mode at about 10 per cent above the polymorph level, which is similar to that found for the non-malignant cervical tissue that we have examined. It is of interest, however, that this tumour was found to have a hypodiploid chromosome number in the region of 38–40. Several cases of lack of proportionality between chromosome number and DNA content in tumours have been noted in our study, and it is hoped to publish further details elsewhere.

In conclusion, the study of the DNA content of a sample of cells from a tumour enables a basic modal DNA value to be established for that tumour ; our evidence suggests that this modal value frequently differs from the normal, thus supplementing the evidence obtained from animal tumours which suggests that each tumour has its stem-line cell which frequently differs from the homologous normal diploid cell in its chromosomal characteristics. A number of questions may be posed which can only be answered by further studies : for instance, whether the modal DNA value varies in different parts of the same tumour or as between a primary tumour and its secondary deposits ; whether there is any correlation with e.g. histological features, clinical course or response to radiotherapy. It is proposed to discuss some of these problems in the succeeding paper, when further observations on cases of carcinoma of the cervix uteri, and the changes that take place during regression after radiation treatment, will be described.

SUMMARY

1. The DNA content of individual cells of human normal and malignant tissues were estimated by a microspectrophotometric method after Feulgen staining.

2. While normal polymorphonuclear leucocytes, lymphocytes, plasma cells and fibroblasts were found to have almost identical amounts of DNA, two specimens of non-malignant tissue from the cervix uteri and two of endometrium each gave modal values which were about 10 per cent higher than the mean of the leucocytes present in the same specimen. Apart from a few cells with doubled DNA content in the epithelial tissues, these cell-types showed little variation in any one sample.

3. In contrast, 17 tumour samples from 12 different sites showed considerable variation in their DNA content. From the frequency histogram for each tumour, a " basic " modal DNA value was derived. As a standard of comparison, the mean DNA value of a sample of the polymorphonuclear leucocytes present in the tumour was determined.

4. The basic modal values of the tumours fell into two approximately equal groups : (i) 10–30 per cent above the control polymorph value ; (ii) in the region of twice the polymorph value. In one tumour the basic DNA value was 10 pe.

cent above the control polymorph value (i.e. equal to that of the corresponding normal tissue), while the basic values of the remaining tumours were greater than 10 per cent above the control polymorph value.

5. The results are discussed in relation to variation in chromosome complement ; the presence of a modal DNA value in tumours, which frequently appears to be different from that of the homologous normal tissue, is considered in relation to the stem-line concept of tumour growth.

We would like to thank Prof. B. W. Windeyer and Prof. J. T. Randall, F.R.S. for encouragement and facilities, Dr. H. B. Fell, F.R.S. and Dr. A. Glücksmann for advice, and our fellow workers at King's College for discussion. Thanks are also due to the members of the staff of Mount Vernon Hospital who made available the material used in this study ; to Dr. K. T. Weavers for the histological reports ; and to Mr. D. Doxey who helped in the preparation of the diagrams. We also wish to acknowledge the financial support given to us by the British Empire Cancer Campaign.

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SEPARATION OF 3,4-BENZPYRENE METABOLITES BY PAPER PARTITION CHROMATOGRAPHY

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THE induction of tumours with 3,4-benzpyrene and other polycyclic hydrocarbons is well known and investigations have been made of the rate at which these substances disappear from the animal body after injection or painting. 8- and 10-monohydroxy-benzpyrene and their corresponding quinones have been isolated from the faeces of rats and rabbits after treatment with benzpyrene (Berenblum and Schoental, 1943; Berenblum, Schoental, Holiday and Jope, 1946).

Four fluorescent metabolites of benzpyrene were separated from the liver, bile, small intestine and faeces of treated animals by Doniach, Mottram and Weigert (1943), Weigert and Mottram (1946*a*; 1946*b*), and Weigert, Calcutt and Powell (1947). These fluorescent metabolites differed in their solubility in organic solvents, in their behaviour on column chromatography and in their fluorescence and absorption spectra.

Recently it was shown that following intravenous injection of benzpyrene, metabolites of this compound are present in the serum of animals in association with the albumin fraction (Chalmers, 1955) and an examination has been made of the nature of these metabolites using paper partition chromatography. A preliminary report of this work has been made (Chalmers, 1956). The present paper relates chiefly to experiments with mice, but reference has been made to experiments of the same nature with other animals.

Although the faeces of animals have been the chief source of benzpyrene metabolites several workers have investigated the urine of animals under test. Elson, Goulden and Warren (1945) who injected rats intraperitoneally with solutions of aromatic hydrocarbons in arachis oil found that in the case of benzpyrene there was no increase in urinary sulphur excretion but a small increase in the level of glucuronic acid. Using radioactive sulphur Gutman and Wood (1950) also found no evidence of the conjugation of benzpyrene with tissue sulphur. Heidelberger and Weiss (1951) however found that following the intravenous injection of benzpyrene-5-C^{14}, 17·3 per cent of the radioactivity was recovered in the urine.

EXPERIMENTAL

Mice were injected intravenously with 0·6 to 1·0 ml. of aqueous colloid containing 0·1 to 0·2 mg. benzpyrene. Rats and fowls were injected with about 1 ml. colloid per 50 g. and per 100 g. body weight respectively. The rabbit to which reference is made in this paper weighed 1750 g. and received orally about 120 mg. of benzpyrene as a solution in arachis oil over a period of 5 days.

Except in the case of the rat, bile removed *post mortem* from the gall bladder was applied to Whatman No. 1 filter paper either directly or as a solution in a mixture of methanol, butan-1-ol and water (1 : 1 : 1). The fluorescent material in the sample was fractionated by ascending paper chromatography using as developer either water-saturated phenol or a mixture of butan-1-ol, acetic acid and water (4 : 1 : 5). The liver and small intestine of the mouse were homogenised in the solvent mixture and the extract after concentration to low volume *in vacuo* was applied to Whatman 3 MM. filter paper and chromatographed as above.

Fluorescence spectrum analysis.—Fluorescence spectra were recorded on a Hilger E3 spectrograph using Ilford HP3 hypersensitive panchromatic plates. Eluates of the fluorescent zones were concentrated to dryness *in vacuo* and the residue dissolved in methanol or the methanol, butanol, water solvent mixture. In either solvent benzpyrene showed the same fluorescence spectrum. Examination of the zones on the chromatographic paper itself as suggested by Dr. P. R. Peacock was also made. This provided a convenient and rapid method of comparing zones having a blue or violet fluorescence. The region of the spectrum below 4000 Å was however obscured by reflected ultraviolet and in the case of benzpyrene applied to paper for reference purposes the spectrum varied according to the solvent in which the hydrocarbon had been dissolved before application to the paper.

Absorption spectrum analyses were carried out on the Hilger Uvispek Spectrophotometer.

Glucuronic acid was determined by the modification of the Tollens' naphthoresorcinol reaction described by Hanson, Mills and Williams (1944). D (+)-glucurone was used as a reference compound and the low standard containing 2·5 μg. gave a definite colour with the reagent.

RESULTS

It was found that the viscosity of the bile varied according to the length of time the mouse had fasted before being killed. After fasting overnight the bile was concentrated but after a few hours' fast the gall bladder was full and more readily handled. The bile of a mouse killed $\frac{1}{4}$-hour after injection did not contain much fluorescent material but after about $\frac{1}{2}$-hour the concentration of fluorescent material appeared to be near the maximum obtained in this type of experiment. The appearance on the chromatogram of the fluorescent metabolites found in bile $\frac{1}{2}$ to $1\frac{1}{2}$ hours after injection of benzpyrene is shown in Table I.

TABLE I.—*Appearance in U.V. of Fluorescent Zones of Chromatogram of Mouse Bile*

Zone.	Rf.	Nature of zone.	Intensity of violet fluorescence.	Fluorescence spectra.	
1	0·1	Narrow	+++	4170Å	4420Å
2A	0·25	„	+	—	—
2B	0·6–0·7	Wide	++	4250	4520
3	0·7–0·8	„	+++	4120	4380
Unchanged benzpyrene if present	0·9–1·0	„	+++	—	—

Zones 1 and 3 appeared to be the major bands. The chromatograms of bile samples after 45, 60 and 90 minutes showed similar zones but the later samples had more zones with a low fluorescence intensity. After 2 hours, although the same pattern was visible on the chromatogram, the concentration of the fluorescent zones was reduced. This decrease was more apparent after 5 and 6½ hours. A sample collected 18 hours after injection showed no fluorescent zones.

Mouse liver.—15-minute sample : there was little fluorescent material of low Rf value and the major fluorescent fraction was zone 3. Unchanged benzpyrene was present. 30-minute sample : zone 1 showed low intensity and zone 3 a high intensity of fluorescence. Unchanged benzpyrene present. 45-, 60- and 90-minute samples : zones 1 and 3 similar to 30-minute sample.

The liver samples showed the presence of straw-coloured fluorescence bands of low and high Rf which appeared to be normal constituents.

Small intestine.—Samples were examined at intervals of 25, 35, 45, 65 and 90 minutes after injection. The appearance of the benzpyrene metabolites on the chromatograms was similar to that of bile samples but in the 90-minute sample additional violet fluorescent zones of low Rf values were present. These bands however had a low intensity of fluorescence. Unchanged benzpyrene was present in the first two but not in later samples. A yellow fluorescent zone of low Rf value was present in all samples and appeared to be a normal constituent.

Large intestine.—Chromatographic examination was made of an extract of large intestine taken 2 hours after injection. This showed the presence of fluorescent material moving with the solvent front in the same manner as the unconjugated hydroxybenzpyrenes found in the faeces. Fluorescent zones of low Rf value seen in the chromatograms of the small intestine were absent or present only in small amount. This indicates the hydrolysis of conjugates of benzpyrene metabolites in the gut.

Fluorescence spectrum analysis.—Zones 1, 2B and 3 of the chromatograms of fluorescent bile from mice injected with benzpyrene colloid were eluted from the paper and concentrated to a suitable volume for fluorescence spectrum analysis. The fluorescence spectra are recorded in Table I. It was found that the fluorescence spectra of zones 1, 2B and 3 of bile collected from mice killed at varying time intervals as specified above were the same. This finding held for the chromatographic zones of samples of small intestine and liver of injected mice.

Zone 1 separated from all the extracts of liver and small intestine in this series of experiments with mice was eluted, pooled, concentrated *in vacuo* and rechromatographed in the same solvent system. A corresponding zone of similar Rf value was obtained. A similar result was obtained with the pooled 2B zones. In the case of the pooled zone 3, some decomposition appeared to take place during extraction and on rechromatography a certain amount of coloured material separated. Most of the zone 3 however was rechromatographed unchanged.

Absorption spectrum analysis.—An analysis has been made of the rechromatographed zones 1 and 3 in the experiments with mice referred to above. The zone 1 sample contained spectroscopically interfering substances but inflections were obtained at : 2870, 2970, 3640, 3840 and 3940Å. 8-Methoxybenzpyrene (Berenblum, Schoental, Holiday and Jope, 1946) has peaks at 2950, 3100, 3630, 3850 and 3950 Å. The methoxy group is similar to the hydroxy in its spectral effect since both have unshared electron pairs on the oxygen atom. The inflections of zone 1 are similar to the peaks of 8-methoxy- or hydroxy-benzpyrene at

3630, 3850 and 3950 Å but one is 10 Å more and the other two 10 Å less and therefore not identical. It is possible that another metabolite is present causing a displacement of the 3650 Å peak.

A characteristic absorption spectrum was not obtained from the examination of mouse zone 3 possibly due to its instability and the small quantity of material present. An absorption spectrum has however been obtained of a rechromatographed zone 3 fraction of rabbit urine from an animal receiving benzpyrene orally. The urine was fractionated by methods similar to those described above. The absorption spectrum of zone 3 corresponded as shown in Fig. 1 with that of 10-methoxy- or 10-hydroxy-benzpyrene.

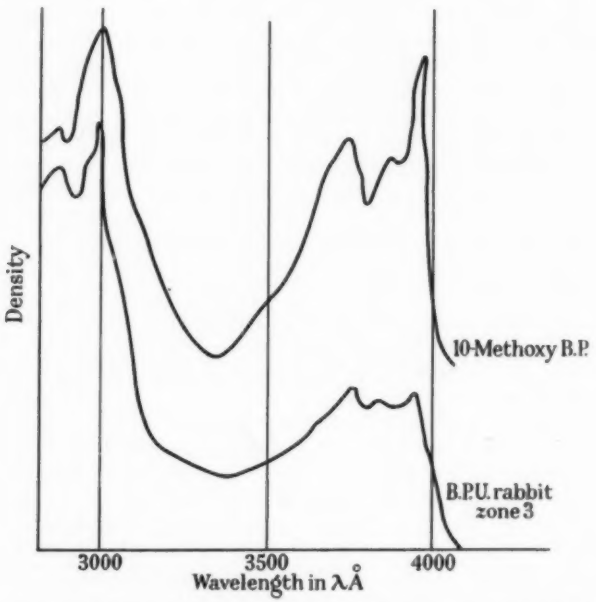

FIG 1.—Absorption spectrum of 3 : 4-benzpyrene metabolite from rubbit urine (zone 3) compared with that of 10-methoxy-3 : 4-benzpyrene (Berenblum *et al.*, 1946).

Glucuronide analysis.—In some early experiments with mice, after injection of benzpyrene, eluates of the fluorescent zones of a chromatogram were tested for the presence of glucuronic acid. In one experiment with mouse small intestine zones 1, 2A and 2B gave positive results while zone 3 gave a negative test. The negative result may have been due to the small quantities present and positive results have been obtained with zone 3 in the case of fowl bile, rat serum and rat and rabbit urine. Rechromatographed samples of zones 1, 2B and 3 of rabbit urine all gave a positive test.

Parallel chromatograms were developed with fluorescent fowl bile and control bile. Corresponding areas were eluted and tested in the usual way. Zones 1 and 3 of the fluorescent bile gave positive results while the control bile gave negative

results. While the results are not conclusive they at least suggest an association of the fluorescent zones with glucuronic acid.

It is hoped to continue these experiments with larger quantities of metabolites from rabbit urine obtained from animals fed with the hydrocarbon in the diet.

SUMMARY

A separation of the fluorescent metabolites of benzpyrene has been made by paper partition chromatography. Mice were injected intravenously with the hydrocarbon and samples of liver, bile and intestine were tested in this way. There appear to be two major constituents, one of low, the other of high Rf value. Preliminary tests indicate that one of these fractions may be a glucuronide conjugate of monohydroxybenzpyrene.

This type of separation has been carried out with the bile and urine of other animals with which similar results to those found in the mouse have been obtained.

The author is indebted to Dr. P. R. Peacock for helpful discussion, and to Mrs. R. H. Jack for the absorption and fluorescence analyses.

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THE METABOLISM OF NEOPLASTIC TISSUES: CARBON DIOXIDE PRODUCTION FROM SPECIFICALLY ^{14}C-LABELLED GLUCOSE BY NORMAL AND NEOPLASTIC TISSUES

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FOLLOWING the pioneer investigations of Warburg and Dickens, and more recently those of Horecker, Calvin and Racker and their associates, the existence of an alternative pathway as distinct from the classical glycolysis, is now well established for a number of normal animal and plant tissues (Racker, 1954; Dickens, 1956). This route is generally called the hexose mono-phosphate (HMP) oxidative pathway and involves a number of reactions in which hexose, pentose, heptose and triose phosphates play a part.

Bloom and Stetten (1953) were the first to use specifically labelled glucose to study the extra-glycolytic breakdown of glucose in normal tissue, especially the liver. Subsequently Abraham, Hirsch and Chaikoff (1954) and others have followed this line of investigation. Regarding neoplastic tissue the information obtained by the tracer technique was scarce (Lewis *et al.*, 1954) when the present work was started. The enzymatic studies by Glock and McLean (1954), however, had provided data which pointed to a general significance of the HMP oxidative pathway in tumour metabolism. In the meantime a number of papers on this subject have appeared (Agranoff, Brady and Colodzin, 1954; Barron, Villavicencio and King, 1955; Abraham, Hill and Charkoff, 1955, Abraham, Cady and Chaikoff, 1956; Wenner, Bloch-Frankenthal and Weinhouse, 1956; Kit, 1956; Kit and Graham, 1956).

In the present communication, data are reported on the production of $^{14}CO_2$ from glucose-1-^{14}C (G-1-^{14}C), glucose-6-^{14}C (G-6-^{14}C) and uniformly labelled glucose (G-u-^{14}C) by many tumours of widely different origin. The results demonstrate a marked difference in the rate of metabolism between the carbon atoms of glucose. Some of these data have already been reported in a preliminary note (Emmelot, Bosch and van Vals, 1955). The investigations have been extended to include a study of the effect of the citric acid cycle inhibitor malonate and of the glycolytic inhibitor monoiodoacetate on the carbon dioxide production from the specifically labelled glucose molecules. Details on the incorporation of ^{14}C into proteins, nucleic acids, fatty acids, cholesterol and lactic acid will be reported later.

MATERIALS AND METHODS

Glucose 1-^{14}C and glucose-u-^{14}C were obtained from the Radio-chemical Centre (Amersham, England), the glucose-6-^{14}C on allocation of the United States Atomic Energy Commission from Dr. H. S. Isbell, National Bureau of Standards (Washington, U.S.A.). The tumours studied were mainly transplanted mouse

tumours ; the spontaneous mammary carcinomas and hepatomas were supplied by Dr. O. Mühlbock of this Institute, to whom our thanks are due.

A fibrosarcoma of the mamma from a female patient was kindly provided by Dr. E. A. van Slooten, surgeon of the Clinic of the Antoni van Leeuwenhoek-Huis. On removal, the tissue was directly placed on crushed ice and used within half an hour. Normal tissues studied were those of white rats of the inbred strain R Amsterdam.

One gram of slices was incubated at 37°C during one hour, unless otherwise stated. The medium consisted of 5 ml. of Krebs-Ringer phosphate buffer (pH 7·4) containing 3 mg. of the labelled glucose. The total activity of each of the three ^{14}C-glucoses amounted to 20 × 5500 counts per minute (see below). Incubation flasks of 50 or 70 ml. provided with a centre well and closed with a rubber serum bottle cap were used. The gas phase was 100 per cent O_2. At the end of the experiment 0·5 ml. 1 N NaOH was injected through the rubber cap into the centre well and 0·2 ml. 0·5 N HCl into the medium. After 4–5 hours a layer of toluene was injected into the centre well, the caps were removed and the contents of the centre well were transferred to a $BaCl_2$ solution. The resulting $BaCO_3$ was weighed and assayed for radioactivity with an end-window Geiger-Müller counter. The radiochemical yield (also called the percentage ^{14}C recovery) R, was calculated as 100 × total counts in the $BaCO_3$ of the respiratory $^{14}CO_2$: total counts in the ^{14}C-glucose administered. Total counts were calculated by multiplying specific activities with the amount of $BaCO_3$ recovered (in mg.). Specific activities are given as the counts per minute measured for an " infinitely" thick layer of $BaCO_3$ under our standard conditions (1·1 square cm. area). Correction was made for self-absorption if necessary. Since 3 mg. glucose was used in each experiment, a recovery of 20 mg. $BaCO_3$ of specific activity 5·5 × 10^3 would represent a 100 per cent yield.

RESULTS AND DISCUSSION

(a) *Incubation of tissue slices with specifically labelled glucose ; effect of time*

The use of specifically labelled glucose is based upon the consideration that the carbon dioxide originating from the breakdown of glucose is initially richer in carbon atom 1 than in carbon 6 if the oxidative pathway is operative. Under the latter condition, the early decarboxylation of 6-phospho-3-ketogluconate yields CO_2 corresponding with carbon 1 from glucose, whereas the sugar skeleton has to undergo many more metabolic transformations before its sixth carbon atom appears in the CO_2 from the citric acid cycle oxidations. Thus, if Rn denotes the radioactivity recovered in the $^{14}CO_2$ from G-n-C^{14} (G = glucose ; n-position labelled with ^{14}C), the ratio R6 : R1 will be smaller than unity. The carbon atoms 1 and 6 are, however, expected to appear in equal rates in the carbon dioxide if the glycolytic scheme is the only route of glucose breakdown (R6 : R1 = 1).

Taking the R6 : R1 ratio as a criterion it has been found that in all the tumours selected for the present study, the HMP oxidative pathway did participate in the breakdown of glucose. The pertinent data are collected in Table I ; the tumour material included spontaneous and transplanted mammary carcinomas of the mouse, fibroadenomas of the rat mamma, a fibrosarcoma of the human mamma, lymphosarcomas of the mouse, interstitial cell carcinomas of the mouse

testis, spontaneous and transplanted hepatomas of the mouse, adrenal cortex carcinomas, granulosa cell and sarcomatoid tumours of the mouse ovary and various mouse sarcomas.

TABLE I.—*Incorporation of Carbon Atoms* 1 *and* 6 *of Specifically Labelled Glucose into Carbon Dioxide.*

Incubation with 1 g. of slices for 1 hour at 37° C; 5 ml. Krebs-Ringer phosphate buffer (pH 7·4), 3 mg. labelled glucose.

Tumour.	Substrate G-n-C^{14} (n).	Rn (per cent).	R6/R1.
Spontaneous mammary carcinoma (C_3H)	6	7·2 2·8	0·43 0·54
	1	16·9 5·2	
Spontaneous mammary carcinoma (DBa)	6	16·1 3·9	0·70 0·60
	1	23·0 6·5	
T 49985—mammary carcinoma*	6	3·3 3·7 3·9	0·47 0·44 0·47
	1	7·1 8·4 8·2	
	6	3·7 2·6 2·4	0·42 0·37 0·32
	1	8·7 6·8 7·4	
T 1014—fibro adenoma mamma rat	6	1·8 2·8	0·51 0·52
	1	3·5 5·4	
Fibro sarcoma mamma (human)	6	0·1 —	0·14 —
	1	0·7 —	
„ „ sclerotic part	6	0·2 —	0·40 —
	1	0·5 —	
T 86157—lymphosarcoma	6	4 4·1	0·23 0·24
	1	17·5 16·7	
T 26473—hepatoma	6	0·86 0·92 1·8	0·36 0·35 0·49
	1	2·4 2·6 3·7	
Spontaneous hepatoma† "early stage"	6	0·27 0·44	0·37 0·46
	1	0·73 0·95	
"Fully developed"	6	0·37 0·11	0·31 0·25
	1	1·18 0·45	
T 5358—interstitial cell carcinoma testis	6	4·5 4·4	0·68 0·86
	1	6·7 5·1	
T 5441—granulosa cell tumour ovary	6	1·8 2·6	0·69 0·70
	1	2·6 3·7	
T 26567—sarcomatoid ovarian tumour	6	5·7 5·1	0·56 0·49
	1	10·1 10·3	
UV 256—sarcoma	6	4·6 3·1 2·7	0·68 0·65 0·43
	1	6·7 4·8 6·3	
T 17572—adrenal cortex carcinoma	6	2·3 3·2	0·60 0·72
	1	3·7 4·4	

* Tumour transplanted from a spontaneous mammary carcinoma of a DBa female.

† Obtained from 2-year-old female (C_{57} Black × C_3He) hybrids; early stage: small nodules, fully developed hepatomas weighed from 2–10 gm.

One of the smallest R6 : R1 ratios was found in experiments with the lymphosarcoma T 86157. Since the results of the tracer experiments may be influenced by the time during which incubation is carried out, an expectation which stems from the very nature of this experimental procedure employed, the effect of the time of incubation on the R6 : R1 and the Ru : R1 ratios has been studied. The results of such an experiment, using T 86157, are illustrated in Table II ; the specific activities (S.A.) of the barium carbonate prepared from the respiratory $^{14}CO_2$ which was collected during 0·5, 1, 2 and 3 hours are given in Fig. 1. The

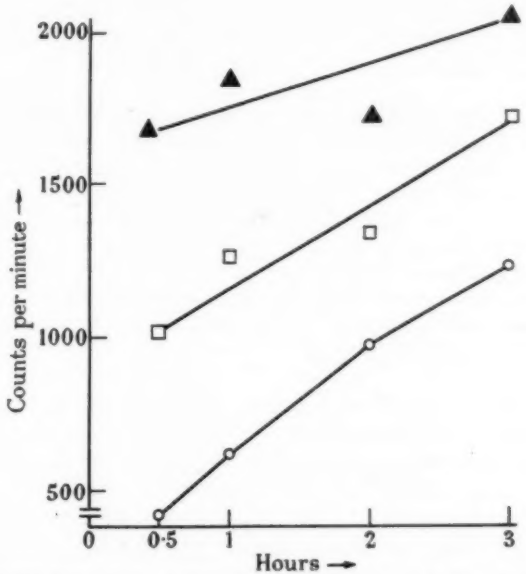

FIG. 1.—Relationship between time of incubation and specific activities of $BaCO_3$ prepared from the $^{14}CO_2$ produced from G-1-^{14}C, G-u-^{14}C and G-6-^{14}C by slices of the transplanted lymphosarcoma T86157. Nineteen small-sized tumours were sliced and from the resulting pooled material twelve portions of 1 g. of slices were each incubated with 3 mg. labelled glucose ($5·5 \times 10^3$ c.p.m.).

○——○ G-6-^{14}C.
□——□ G-u-^{14}C.
▲——▲ G-1-^{14}C.

S.A. of the $^{14}CO_2$ from G-1-^{14}C was initially much higher than that from G-6-^{14}C (1726 against 436 counts per minute after 30 minutes' incubation) but with longer times of incubation the curve representing the S.A. of the latter rose more sharply than that of the former (to 2087 and 1168 counts per minute after 3 hours, respectively). Both the initial level of radioactivity in the $^{14}CO_2$ and the time course of $^{14}CO_2$ production in the two cases were consistent with the theoretical expectation assuming that a direct pathway of glucose oxidation such as that formulated for the HMP " shunt " should be operative in addition to the citric acid cycle. The ratios R6 : R1 and Ru : R1 will thus tend to higher values on increasing the time of incubation.

TABLE II.—*Effect of Time on the Incorporation of Carbon Atoms* 1 *and* 6 *from Specifically Labelled Glucose into Carbon Dioxide by Slices of the Lymphosarcoma T* 86157.

1 g. of slices was incubated with 3 mg. ^{14}C-glucose at 37° C.

From :	Radioactivity recovered in $^{14}CO_2$ (per cent) After : 0·5 hour.	1 hour.	2 hours.	3 hours.
G-6-^{14}C . . .	1·39	3·55	8·50	13·8
G-1-^{14}C . . .	6·59	12·3	17·3	24·7
G-u-^{14}C . . .	3·88	7·71	13·8	20·6
Ru : R1 . . .	0·59	0·63	0·79	0·83
R6 : R1 . . .	0·21	0·35	0·49	0·56

Table III illustrates the results of a similar experiment performed with slices of rat heart. In this case the S.A. of the $^{14}CO_2$ from both G-1-^{14}C and G-6-^{14}C rise concurrently with time. Although small differences in the R6 : R1 ratios with time were consistently observed in the latter experiments, the data did not permit the conclusion that the HMP oxidative pathway was present. The latter might perhaps be made apparent if the citric acid cycle oxidations were blocked by adding an inhibitor such as malonate, because such a procedure should result in a situation somewhat resembling very short times of incubation. Addition of malonate to study the conversion of labelled glucose in liver was applied by Agranoff, Colodzin and Brady (1954*b*).

TABLE III.—*Effect of Time on the Incorporation of Carbon Atoms* 1 *and* 6 *of Specifically Labelled Glucose into Carbon Dioxide by Slices of Rat Heart.*

1 g. of slices were incubated with 3 mg. ^{14}C-glucose (see Methods : specific activity $5·5 \times 10^3$ c.p.m.) at 37° C.

Time (hours).	G-n-^{14}C (n).	$BaCO_3$ (mg.).	$BaCO_3$ (c.p.m.).	Rn (per cent).	R6/R1.
1	6	10·1	740	6·79	0·93
	1	10·0	810	7·36	
2	6	12·9	1174	13·8	0·99
	1	12·9	1200	14·1	
3	6	18·2	1694	28·0	1·05
	1	17·9	1694	26·7	

(b) *Effect of malonate on the* ^{14}*C recoveries in carbon dioxide*

In the case of a tissue in which the HMP oxidative pathway was completely absent, there should be no difference in the S.A. of, or in the total recovery of isotope in, the $^{14}CO_2$ derived from G-1-^{14}C and G-6-^{14}C ; the addition of malonate would here be expected to cause a lowering of both S.A.'s to the same extent. If, on the other hand, the fall in the S.A. of the $^{14}CO_2$ from G-1-^{14}C as a result of malonate addition should be less than that observed for the $^{14}CO_2$ from G-6-^{14}C, this would mean that the extra-glycolytic pathway had revealed itself in consequence of the inhibition of the citric acid cycle.

The latter was actually found true with several normal rat tissues such as heart, kidney, brain and diaphragm, which yielded R6 : R1 ratios of approximately

unity when incubated in the absence of malonate for various periods. When malonate (0·03 M) was added, the R6 : R1 ratios were significantly smaller than unity as can be seen from typical examples listed in Table IV.

TABLE IV.—*Effect of Malonate on the Incorporation of Carbon Atoms* 1 *and* 6 *from Specifically Labelled Glucose into Carbon Dioxide by Slices of Normal Rat Tissues.*

Incubation with 3 mg. of ^{14}C-glucose (specific activity $5·5 \times 10^3$ c.p.m.). Slices of the organs from several rats were pooled before transfer to the four incubation flasks of each experiment. Malonate 0·03 M.

		Malonate absent.			Malonate present.		
Tissue mg. per flask.	Incubation period (hours).	$Ba^{14}CO_3$ from G-n-^{14}C counts per minute. n=6.	n=1.	^{14}C-recovery (per cent) as R6/R1.	$Ba^{14}CO_3$ from G-n-^{14}C counts per minute. n=6.	n=1.	^{14}C-recovery (per cent) as R6/R1.
Heart (650)	3	1794	1831	20·5/19·6=1·05	357	808	1·56/3·76=0·42
Kidney (1000)	3	1393	1581	80·0/79·0=1·01	372	663	8·48/16·4=0·52
Diaphragm (600)	2	979	1005	6·59/6·76=0·98	370	480	1·28/1·71=0·76
Brain (1000)	1	1428	1365	19·6/18·1=1·08	262	597	1·76/3·79=0·46
		n=u.		Ru.	n=u.		Ru.
Diaphragm (1000)	1	586		2·1	600		0·6
Brain (1000)	1	2730		24·0	2036		9·8
	2	2782		39·2	1919		12·4
Heart (500)	1	1045		5·5	528		1·3
Kidney (500)	1	1700		45	1300		20

It may thus be concluded that these tissues are dependent upon the citric acid cycle for oxidation of carbon atom 1 of glucose and that the HMP oxidative pathway does not appear to be operative to such an extent as to be demonstrable by the tracer experiments. By inhibiting the cycle, however, the HMP oxidative pathway becomes manifest, although, as judged from the total amount of isotope recovered, the latter can only account for a small fraction of the C_1 atoms of glucose normally oxidized.

When the S.A.'s of the $^{14}CO_2$ produced from G-u-^{14}C, with or without malonate present, were compared, in general no significant fall was noted (Table IV, lower part). Since the quantity of $BaCO_3$ recovered in the presence of the citric acid cycle block was always less, the total amount of isotope recovered in the $^{14}CO_2$ was also markedly less than that found in the absence of malonate. The fact that the S.A. of the $^{14}CO_2$ originating from G-u-^{14}C remained of the same order despite the presence of malonate, might have been due to the decarboxylation of the uniformly labelled pyruvate yielding acetylcoenzyme A (followed by formation of acetoacetate). Perhaps a recycling of the labelled substrate through the pentose cycle (de la Haba, Leder and Racker, 1955) must also be kept in mind.

Next, a number of tumours and lung tissue, in which the HMP oxidative pathway had been found to be operative,* were studied in the presence of malonate.

* Rat liver (R6 : R1 = 0·20) and spleen (R6 : R1 = 0·60) belong to this type of tissue also.

The S.A. of the $^{14}CO_2$ produced from G-1-^{14}C may be expected to rise under the latter condition, as compared with that collected in the absence of malonate, since the isotope " dilution " by way of the citric acid cycle is prohibited. On the other hand, the ^{14}C-content of the carbon dioxide, originating from G-6-^{14}C, will fall, the citric acid cycle in this case being the only route by which radioactive CO_2 can be produced.

The data of Table V show that the latter is invariably found to be true. In the presence of malonate, practically no radioactivity appeared in the carbon dioxide which was produced from G-6-^{14}C by slices of the sarcoma UV 256, the lymphosarcoma T 86157 and the ovarian granulosa cell tumour T 5441, indicating that the citric acid cycle oxidations were almost completely blocked. The carbon dioxide formed in the presence of malonate from G-6-^{14}C by slices of the testicular tumours (T 5358 and T 26554) and the spontaneous mammary carcinomas of DBa female mice contained more ^{14}C than the CO_2 produced by the slices of the former group of tumours. This suggests that in the latter tumours oxidation through the citric acid cycle had still taken place, although on a much smaller scale than in the absence of malonate, as judged from the total amount of isotope recovered.

The S.A. of the $^{14}CO_2$ from G-1-^{14}C in the presence of malonate was higher than in its absence in experiments with four of the tumours (T 5358, UV 256, T 86157 and T 5441) ; this was not the case with two other tumours (the mammary carcinoma and T 26554) and normal lung tissue. However, the S.A. of the $^{14}CO_2$ formed from G-1-^{14}C in the presence of malonate by the latter three tissues never

TABLE V.—*Effect of Malonate on the Incorporation of Carbon Atoms* 1 *and* 6 *from Specifically Labelled Glucose into Carbon Dioxide by Slices of Transplanted Mouse Tumours and Lung Tissue.*

1 g. of slices was incubated for 1 hour at 37° C. with 3 mg. ^{14}C-glucose (specific activity $5{\cdot}5 \times 10^3$ c.p.m.).

	Malonate absent.			Malonate present.		
Tissue.	$Ba^{14}CO_3$ from G-n-^{14}C counts per minute. n=6.	n=1.	^{14}C-recovery (per cent) as R6/R1.	$Ba^{14}CO_3$ from G-n-^{14}C counts per minute. n=6.	n=1.	^{14}C-recovery (per cent) as R6/R1.
T 5358	887	1629	6·0/11·7=0·51	296	2760	1·3/10·0=0·13
,,	594	1234	3·8/7·9=0·48	156	1904	0·8/9·3=0·09
T 26554	1236	1766	8·6/11·9=0·72	376	1513	2·3/9·2=0·25
Spontaneous mammary carcinoma (DBa)	814	1493	3·94/6·5=0·60	285	1229	0·99/5·46=0·18
UV 256	628	1282	4·0/6·9=0·58	35	1389	0·1/5·0=0·02
T 86157	470	2365	2·6/14·6=0·18	39	3452	0·07/8·5=0·008
,, *	506	2794	2·8/11·7=0·21	69	3535	0·2/11·8=0·01
T 5441	493	1071	1·3/3·3=0·40	0	1397	0/2·0=0
Lung†	1706	2977	27·7/46·5=0·57	485	2217	6·26/25·0=0·25
	n=u.		Ru.	n=u.		Ru.
T 86157*	1433		6·1	2116		6·3
T 5441	1222		5·8	1576		4·9
T 5358	1440		8·0	1521		6·8

* Same experiment.
† Incubated during 3 hours.

showed such a drop as was found when the tissues listed in Table IV were incubated in the presence of malonate.

Consequently, the total recovery of isotope in the $^{14}CO_2$ produced from G-1-^{14}C was not at all or only slightly diminished following the inhibition of the citric acid cycle of all the tumours studied. The same was found for G-u-^{14}C.

This stands in contrast to what was found with the normal tissues listed in Table IV, which were much more dependent upon the citric acid cycle for oxidation. In comparing these results it should also be noted that in experiments with the latter tissues, malonate reduced the quantity of CO_2 (recovered as mg. $BaCO_3$) to a greater extent than in the experiments with the tumours.

(c) *Effect of monoiodoacetate (MIA) on the ^{14}C recoveries in carbon dioxide*

Dickens and Glock (1951) have shown that iodoacetamide in a concentration of 0·01 M had very little effect on the activities of the glucose 6–phosphate and 6-phosphogluconate dehydrogenases of liver. The formation of sedoheptulose, an intermediate of the HMP oxidative pathway, from glucose 6-phosphate and triphosphopyridine nucleotide was not affected in the presence of MIA ($5{\cdot}4 \times 10^{-4}$M) in our experiments with soluble enzyme preparations from the tumours (Bosch, van Vals and Emmelot, 1956). The formation of sedoheptulose from glucose in tumour slices incubated in the presence or absence of MIA could not be demonstrated by spectrophotometric means. In the presence of MIA, hexose concentration fell off very slowly and triose accumulated as shown by the anthrone reaction ; in addition the formation of fructose could be demonstrated by means of the cysteine-sulfuric acid method (Bosch, van Vals and Emmelot, 1956). Although no exact quantitative measurements of the intermediates were made, it was nevertheless apparent from the absorption spectra obtained that glucose disappearance was very markedly less than in the absence of MIA. In the presence of MIA, lactic acid formation was also significantly diminished.

As a result of the glycolytic block exerted by MIA, the amount of isotope recovered in the carbon dioxide which was produced from G-u-^{14}C was diminished ; for example, 500 mg. of slices of the testis tumour T 5358 produced 3·1 mg. $BaCO_3$ of 875 counts/min. but in the presence of MIA only 2·6 mg. $BaCO_3$ of 345 counts/min. If the Embden-Meyerhof pathway had been blocked completely at the triose phosphate stage by MIA, any $^{14}CO_2$ recovered should have been formed exclusively by glucose oxidation via the HMP oxidative pathway. However, our experiments are not conclusive with regard to the effectiveness of the MIA block. The experiments with G-u-^{14}C demonstrated that in the presence of MIA relatively more unlabelled than labelled substrate became oxidized as compared with oxidation in the absence of the inhibitor. This is easily understood since the block slows down glucose dissimilation, but still allows endogenous compounds, such as lactic acid and fatty acids to be oxidized through the citric acid cycle. No radioactivity would be present in the $^{14}CO_2$ produced from G-6-^{14}C if the glycolytic pathway was completely blocked in the presence of MIA. It was found, however, that the S.A's of the $^{14}CO_2$ from both G-6-^{14}C and G-1-^{14}C were diminished to about half of the original value. Accordingly the R6 : R1 ratios did not differ much either in the presence or absence of MIA. Thus, either the glycolytic block had been inefficient or a randomization of the tracer from G-6-^{14}C had occurred as a result of which $^{14}CO_2$ was produced by a pathway other than the citric acid cycle. Since triose accumulates as a result of the inhibition by MIA,

an equilibration of tracer via glyceraldehyde 3-phosphate ⇌ dioxyacetone phosphate might easily have taken place. A resynthesis to hexose would then mean that carbon atom 6 of the original glucose had been converted into carbon atom 1 of the newly synthesized hexose. This phenomenon would tend to dilute the S.A. of the $^{14}CO_2$ produced from G-1-^{14}C and in the case of G-6-^{14}C would produce radioactive carbon dioxide through the HMP oxidative pathway. As regards the other possibility of inefficient blocking, when the concentration of MIA was raised ten-fold, the S.A. of the carbon dioxide obtained from only some of the preliminary experiments with G-6-^{14}C, still remained of the same order as the corresponding values of the earlier experiments. Further investigations are being carried out in this connection.

SUMMARY

Normal and neoplastic tissues were incubated with uniformly labelled glucose and glucose specifically labelled in either carbon atom 1 or carbon atom 6. From the recovery of isotope in the carbon dioxide produced, it followed that in all neoplastic tissues studied a marked difference in the rates of metabolism between carbon atom 1 and 6 did exist.

Furthermore, observations on the effect of the time of incubation and of the presence of a citric acid cycle block (malonate) were consistent with the view that the hexose monophosphate oxidative pathway was involved in the glucose metabolism of the tumours. In some experiments the effect of a glycolytic block (monoiodoacetate) was also studied. In a number of normal rat tissues (heart, kidney, brain, diaphragm) the existence of the hexose monophosphate oxidative pathway was only revealed when incubation was carried out in the presence of malonate.

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THE METABOLISM OF NEOPLASTIC TISSUES: DEMONSTRATION OF INTERMEDIATES AND REACTION SEQUENCES OF THE HEXOSE MONOPHOSPHATE OXIDATIVE PATHWAY

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In the preceding communication (van Vals, Bosch and Emmelot, 1956) data were presented which demonstrated that a marked difference exists in the rate of metabolism between the glucose carbon atoms 1 and 6 in tumour tissue. The results indicated that the hexose monophosphate (HMP)* oxidative route might be operative.

In support of this, experiments have been carried out to establish the identity of the intermediates (especially sedoheptulose) and the reactions involved in this pathway. The results obtained with cell-free extracts, homogenates and slices corroborated the previous conclusion regarding the participation of the HMP oxidative pathway in the glucose metabolism of the neoplastic tissues. In the present work tumours of widely different origin were studied and these investigations thus provide information additional to that published since the present work was started (Agranoff, Brady and Colodzin, 1954; Barron, Villavicencio and King, 1955; Wenner, Bloch-Frankenthal and Weinhouse, 1956; Abraham, Hill and Chaikoff, 1955; Abraham, Cady and Chaikoff 1956; Kit, 1956; Kit and Graham, 1956).

METHODS AND MATERIALS

The G6-P and 6-PG dehydrogenases were determined according to Glock and McLean (1953, 1954). Results are given in units of enzyme activity per gram fresh weight of tissue, one unit being defined as the quantity of enzyme which at 20°C and pH 7·4 reduces 0·01 μ. mole TPN per min. Optical densities were read at 340 mμ. in the Quartz Unicam Spectrophotometer (SP500). In some cases the reduced TPN was measured by a coupled oxidation-reduction involving glutathione (Anderson *et al.* 1952; Rall and Lehninger, 1952; Kinoshita and Masurat, 1954). Reduced glutathione was determined according to the nitroprusside method of Grunert and Philips (1951). In other experiments 2 : 6-dichlorophenol-indophenol was used as electron acceptor (Axelrod *et al.*, 1953).

The orcinol reaction of Mejbaum (1939) as modified by Albaum and Umbreit (1947) was used for pentose determination, taking the difference in optical

* Abbreviations used: G6-P = glucose ·6-phosphate; 6-PG = 6-phospho gluconate, R5-P = ribose 5-phosphate; Ru5-P = ribulose 5-phosphate; S7-P = sedoheptulose 7-phosphate; F6-P = fructose 6-phosphate; FDP = fructose 1,6-diphosphate; T3-P = triose 3-phosphate; DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; ATP = adenosine triphosphate; TCA = trichloroacetic acid; MIA = monoiodoacetate.

densities at 660 and 600 mμ. as the basis for the calculation of pentose concentration.

Corrections for the presence of sedoheptulose (the concentration of which was determined independently by the method described below) were applied making use of a standard curve relating the sedoheptulose concentration *versus* the difference in optical density at 600–660 mμ. observed for sedoheptulose in the orcinol reaction (compare also Dische, 1953). These corrections were small.

Heptulose was measured with the cysteine-sulfuric acid (CyRI) procedure of Dische, Shettles and Osnos (1949). Absorption spectra were read at 18 hours after the addition of cysteine (Axelrod *et al.*, 1953). The difference in optical densities between 510 and 540 mμ. was used for the quantitative determination of heptulose. Sedoheptulosan monohydrate served as a standard (Axelrod *et al.*, 1953; Horecker, Smyrniotis and Klenow, 1953).

Spectra in the orcinol and cysteine-sulfuric acid test similar to those described by Newburgh and Cheldelin (1955) were found, although for quantitative purposes the latter cannot be strictly compared with those obtained by the method of Axelrod *et al.* (1953).

The quantitative determination of hexose was accomplished by the use of the anthrone method (Seifter, Dayton, Novic and Muntwyler, 1950) after correction for heptose and triose.

Triosephosphate was measured as alkali labile phosphate (Umbreit, 1947). The molar extinctions of glucose and fructose are equal in the anthrone test (635 mμ.) whereas the molar extinction of fructose is twice that of glucose in the cysteine-sulfuric acid method (420 mμ.).

Paper chromatographic analysis of the various phosphorylated sugars was performed using the solvent system ethylacetate : acetic acid : water (3 : 3 : 1) (Mortimer, 1952; Hauge, King and Cheldelin, 1955). The phosphate esters were located by the method of Hanes and Isherwood (1949). Heptulose was identified on the chromatograms by the orcinol spray, giving the characteristic blue-greenish colour according to Klevstrand and Nordal (1950) (compare Williams and Bevenue 1951).

Ten per cent tumour homogenates were prepared according to Glock and McLean (1953) in KCl-bicarbonate and were freed from nuclei and mitochondria by centrifugation for 15 minutes at 6000 × g. in the " Eispirouette " (Phywé) at 0°C. The supernatant was dialyzed overnight against distilled water in the icebox. No change in results of the experiments in which the dehydrogenases were measured were found when the homogenization of the tissues was varied from 500–2000 r.p.m. for periods of ½–2 minutes.

Tissue slices were cut in an apparatus consisting of rotating circular razor blades (de Flines, 1951) and introduced into Krebs-Ringer phosphate buffer of pH 7·4 (previously chilled in cracked ice) blotted on filter paper and weighed. Half gram aliquots of the pooled slices were transferred to 25 ml. incubations flasks containing 2·5 ml. Krebs-Ringer phosphate and 7·5 μ-mole of either glucose, G6-P or R5-P. The flasks were stoppered and incubated for various times at 37°C. Reactions were stopped by addition of 2·5 ml. of a 10 per cent trichloroacetic acid solution. The resulting mixtures were homogenized and aliquots of the supernatants were used for carbohydrate analysis.

Incubation of the complete tumour homogenates were performed anaerobically according to Novikoff, Potter and Le Page (1948).

Further details are given in the legends of the text figures and tables.

In general all experiments were repeated with as great a number of tumours as available. The tumours of choice were those mentioned in the previous communication (van Vals, Bosch and Emmelot, 1956), especially those in Table I. The experiments with slices and total homogenates are illustrated by typical experiments.

TABLE I.—*The Activities of the Glucose 6-phosphate and 6-phospho-gluconate Dehydrogenases in the Soluble Fractions of Some Transplanted Mouse Tumours.*

The reaction mixture consisted of 0·5 ml. 0·25 M *Tris* (pH 7·6 for both dehydrogenases), 0·5 ml. 0·1 M $MgCl_2$ an appropriate amount of enzyme preparation (0·05–0·2 ml.), 200 μg. TPN in a total volume of 2·4 ml. The reaction was started by the addition of 0·1 ml. 0·5 M substrate. Blanks contained all components except TPN. Incubation at 20°C. Optical measurements were carried out after deproteinization at various times (compare Glock and McLean, 1953). For comparison data on rat liver (inbred strain R Amsterdam) are included.

Tumour.	G6-P dehydrogenase. (Units/g. wet weight of tissue.)	6-PG dehydrogenase. (Units/g. wet weight of tissue.)
Interstitial cell carcinoma of the mouse testis :		
T 5358	410	90
„	360	160
T 65611	450	140
„	320	30
Granulosa cell tumours of the mouse ovary :		
T 5441	360	40
„	460	50
T 26567 (g)	235	35
Luteoma : T 7331	250	150
	280	100
	360	120
	380	100
Sarcomatoid tumour of the mouse ovary :		
T 24202	210	60
„	200	50
Hepatoma : T 28102	110	80
Lymphosarcoma : T 86157	170	115
Liver :		
♀ rat	340	120
♂ „	210	100
♀ „ fasted	360	120
♂ „ „	210	110

D-G6-P (Ba-salt) was purchased from Fluka (Basel, Switzerland), R5-P (Ba-salt) from Nutritional Biochemicals Corporation (Cleveland, U.S.A.), glucose and ribose from Hoffmann-LaRoche (Basel, Switzerland), TPN from Pabst Laboratory (Milwaukee, U.S.A.), DPN (— 95), FDP, triosephosphate from Boehringer U.S. (Mannheim, Germany), glutathione from Light (Colnbrook, England), and ATP from Zellstofffabrik Waldhof (Wiesbaden, Germany). Sedoheptulosan monohydrate (2,7-anhydro-β-D-altro-heptulopyranose monohydrate) was kindly supplied by Dr. N. K. Richtmeyer of the National Institutes

of Health (Bethesda, U.S.A.). 6-PG was prepared from G-6P (Na-salt) according to the method of Seegmiller and Horecker (1951), slightly modified due to insolubility of the barium salt of the latter. The oxidation with bromine was performed in the presence of 0·075M $NaHCO_3$ (pH 7·9) using twice the amount necessary to neutralize the hydrobromic acid produced. The purity of the isolated product was measured enzymatically with a 6-PG dehydrogenase preparation isolated from rat liver according to Glock and McLean (1953). The product contained 67 per cent 6-PG and less than 1 per cent G6-P as assayed in the cysteine-sulfuric acid test.

RESULTS

I. *Experiments with Cell-free Extracts*

Starting with the dehydrogenases, experiments were undertaken to investigate whether the enzymes involved in the HMP oxidative pathway could be demonstrated in extracts prepared from tumours which had been studied with the tracer technique (van Vals, Bosch and Emmelot, 1956). The soluble fractions were prepared after homogenization, centrifugation and dialysis at 0°C.

A. *Glucose 6-phosphate and 6-phospho gluconate dehydrogenase*

Reaction 1 : G6-P + TPN^+ → 6-PG + TPNH + H^+
Reaction 2 : 6-PG + TPN^+ → 3-keto 6-PG + TPNH + H^+

The activities of the dehydrogenases were determined according to Glock and McLean (1953). The activities were calculated from the differences in optical densities at 20°C resulting from G6-P plus 6-PG, and 6-PG addition separately.

Table I summarizes the activities of the enzymes from a number of our tumour strains ; the data are expressed in units of enzymatic activity per gram fresh tissue. The reduction of TPN could also be followed by coupling the TPNH formed with two other enzyme systems which were present in the soluble fractins, *i.e.* the glutathione reductase (acceptor added : oxidized glutathione, Fig. 1a) and the TPNH-diaphorase (acceptor added : 2 : 6-dichlorophenol indophenol, Fig. 1b).

B. *Ribolysis, formation of sedoheptulose and hexose**

(*a*) *Reaction 3*† : R5-P → Ru5-P (pentose isomerase)
Reaction 4 : R5-P + Ru5-P → S7-P + T3-P (transketolase)
Reaction 5 : S7-P + T3-P → F6-P + tetrose-phosphate (transaldolase)
Reaction 6 : Ru5-P + tetrosephosphate → F6-P + T3-P (transketolase)
Reaction 7 : 2 T3-P → FDP (aldolase)

Incubation of the soluble tumour fractions with R5-P at 37°C led to the disappearance of pentose with concommitant formation of sedoheptulose, hexose and triose. These findings can be covered by the reaction sequence described in reactions 3-7. Any or all of the three different ways of hexose formation may be followed (reaction 5, 6 and 7).

* For a review of the reactions taking place in the extra-glycolytic breakdown of glucose compare Racker (1954), Dickens (1956) and Horecker and Mehler (1955).

† Recent investigations (Srere *et al.*, 1955) have revealed that still another reaction may be involved, e.g. the epimerization of Ru5-P to xylulose 5-phosphate (X5-P) by a phosphoketopentose isomerase. In that case Ru5-P of reaction 4 and 6 has to be replaced by X5-P.

Pentose disappearance was followed using the orcinol reaction; Fig. 2, illustrating an experiment with the soluble fraction of the transplanted mouse sarcoma UV 256, shows that concommitantly with the decrease in optical density at 660 mμ, the slopes of the individual curves between 660 and 550 mμ. changed. This change is not only due to the disappearance of pentose, but also to the formation of sedoheptulose, which according to Horecker, Smyrniotis and Klenow (1953) displays a very weak band at 580–600 mμ. in the orcinol reaction.

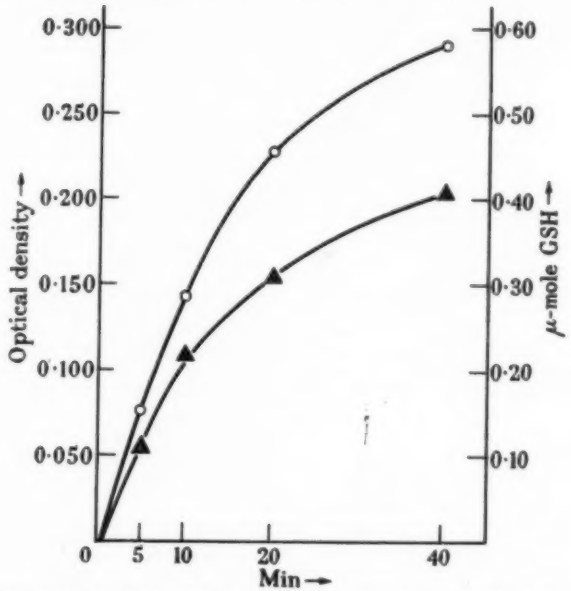

FIG. 1a.—Coupled oxidation-reduction reaction between glucose 6-phosphate, triphosphopyridine nucleotide and glutathione by the soluble fractions of two transplanted mouse tumours.

T 5441 = granulosa cell tumour of the ovary : ▲——▲.
T 24202 = sarcomatoid ovarian tumour : ○——○.

1·0 ml. 0·25 M Tris (pH 7·6), 1·0 ml. 0·1 M $MgCl_2$, 0·2 ml. 0·05 M, G6-P, 1·0 ml. 0·0004 M glutathione (GSSG), 400 μg. TPN in 0·4 ml. water, 0·4 ml. enzyme preparation (dialyzed supernatant from a 10 per cent homogenate). Incubation at 37°C. At various times 1 ml. aliquots were transferred to 3·0 ml. 3 per cent metaphosphoric acid. The resulting mixture was saturated with 1·5 g. NaCl and centrifuged, in 2·0 ml. aliquots of the clear solution. GSH was assayed according to Grunert and Philips (1951).

Since the absorption spectra of glucose, fructose and triose do not show any difference in optical density between 660 and 600 mμ. (Newburgh and Cheldelin, 1955) pentose concentration is directly proportional to the difference in optical densities at these two wave lengths provided that a correction for sedoheptulose is made. The quantitative determination of heptulose was accomplished with the aid of the cysteine-sulfuric acid procedure (compare experimental part). Fig. 3a and 3b serve to illustrate two experiments in which R5-P was found to be converted to heptulose (510 mμ.) and to hexose (420 mμ.) by the soluble prepara-

tions from the sarcoma UV 256 and the ovarian tumour (granulosa cell type) T 5441, respectively.

Quantitative data on the disappearance of R5-P and the formation of S7-P are given in Fig. 4 and 5. A complete analysis of the formation of hexose, sedoheptulose and triose from R5-P after successive times of incubation is presented in Table II. Triose phosphate was measured as alkali-labile phosphate and hexose by the use of the anthrone colorimetric method, corrected for heptulose

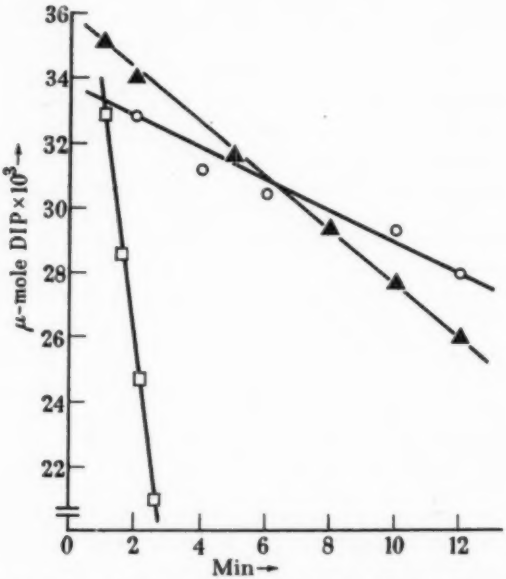

FIG. 1*b*.—Coupled oxidation-reduction reactions between glucose 6-phosphate, triphosphopyridine nucleotide, and 2 : 6-dichlorophenol indophenol by the soluble fractions of some transplanted mouse tumours.

T 5441 = granulosa cell tumour : ▲——▲.
T 24202 = sarcomatoid ovarian tumour : ○——○.
T 5358 = interstitial cell carcinoma of the testis : □——□.

0·5 ml. 0·25 M Tris (pH 7·6), 0·5 ml. 0·25 M $MgCl_2$, 0·1 ml. 0·05 M G6-P, 200 μg. TPN, 25 μg. 2 : 6-dichlorophenol indophenol (DIP) and 0·1 ml. enzyme preparation in a total volume of 2·5 ml. Incubation at 20°C. DIP reduction was read at 610 mμ.

and triose. Results obtained with the paper chromatographic methods, mentioned in the experimental part, confirmed the spectrophotometric data concerning the presence of the various sugar phosphates.

(*b*) Since the various enzymes of the HMP oxidative pathway appeared to be present in the soluble tumour fractions, it should be possible to show in one experiment the synthesis of G6-P from R5-P (reactions 3–6 ; F6-P of reaction 5 or 6 can be converted into G6-P by the action of phosphohexoisomerase, which is also present) followed by an oxidation of G6-P on addition of TPN (reactions 1 and 2).

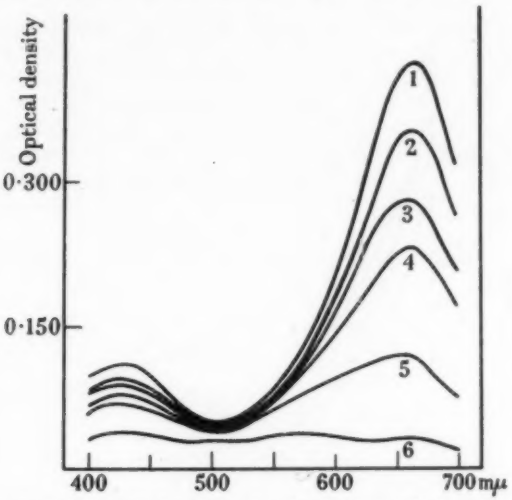

FIG. 2.—Disappearance of ribose 5-phosphate after incubation with the soluble fraction of the mouse sarcoma UV 256, as measured by the orcinol reaction.

The incubation mixture consisted of 0·5 ml. 0·05 M R5-P, 1·0 ml. 0·25 m *Tris* (pH 7·6), 1 ml. 0·1 M $MgCl_2$, 0·4 ml. enzyme preparation ; total volume 5·0 ml. Incubation at 37°C in air. At the times indicated (Curves 1–6 ; 0, 20, 40, 60, 120 and 180 minutes) 0·5 ml. of the incubation mixture was transferred to 4·5 ml. 5 per cent TCA, 0·3 ml. of the resulting deproteinized solution was used for the orcinol reaction.

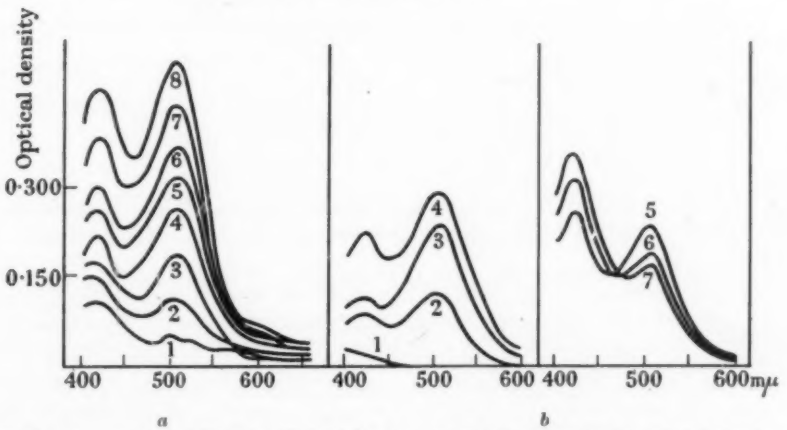

FIG. 3*a*.—Conversion of ribose 5-phosphate to hexose and heptulose by the soluble fraction of the mouse sarcoma UV 256, as measured by the cysteine-sulfuric acid method.

For experimental conditions compare Fig. 2.

Curves 1–8 illustrated the spectra obtained from aliquots (one ml. of the deproteinized solutions was used for the cysteine-sulfuric acid test), taken at 10, 30, 60, 90, 120, 180, 300 and 360 minutes respectively.

FIG. 3*b*.—Conversion of ribose 5-phosphate to hexose and heptulose by the soluble fraction of the granulosa cell tumour of the ovary T 5441, as measured by the cysteine-sulfuric acid method.

Experimental conditions similar to those of Fig. 2, except that 0·2 m. 0·05 M R5-P was used. Curves 1–7 illustrate the spectra obtained from aliquots taken at 0, 30, 60, 120, 180, 240 and 480 minutes respectively.

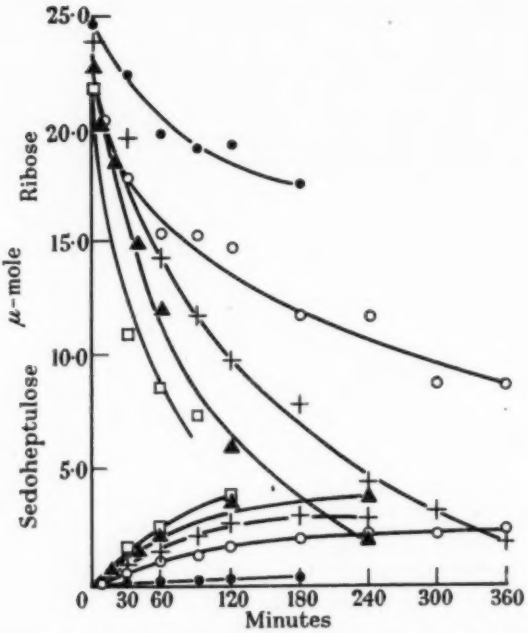

FIG. 4.—Breakdown of ribose 5-phosphate and formation of sedoheptulose by soluble preparations from normal and tumour tissues (long-term experiments).

For experimental conditions compare Fig. 2. Appropriate aliquots of the deproteinized solution were assayed for pentose and heptose.

●——● Rat heart, ○——○ mouse sarcoma UV 256, +——+ mouse hepatoma T 28012, ▲——▲ mouse liver, □——□ mouse lymphocarcoma T 86157.

TABLE II.—*Formation of Heptose, Hexose and Triose from Ribose 5-phosphate by the Soluble Fraction of the Mouse Ovarian Tumour T 5441 (granulosa cell type).*

Tubes containing 0·4 ml. 0·25 M *Tris* (pH 7·6), 0·4 ml. 0·1 M $MgCl_2$, 0·2 ml. 0·05 M R5-P, 0·16 ml. enzyme preparation in a total volume of 2·0 ml. were incubated at 37°C in air for the various times indicated. Reaction was stopped by adding 1·6 ml. of 10 per cent TCA. 2·0, 0·18, 0·05 and 0·18 of the deproteinized reaction mixtures were used for the determination of triose phosphate, the cysteine-sulfuric acid-, the orcinol- and the anthrone reaction, respectively. The blanks contained all components except R5-P.

Compound.	μ-Mole, after (minutes)					
	0.	30.	60.	90.	120.	180.
Pentose . .	10	7·1	6·3	4·8	4·5	3·7
Heptose . .	0	·32	·58	·82	·88	·92
Hexose . . .	0	·20	·40	1·0	1·6	2·2
Triose . . .	0	·60	·67	·76	·85	·78

This was accomplished in the following way: At 60, 120 and 180 minutes after incubation of the soluble fraction of the ovarian tumour T 5441 with R5-P, two 0·5 ml. aliquots were removed each time. To one tube of each pair of samples 200 μg. TPN in 0·1 ml. water was added, the other received water only. Subsequently the tubes were left at room temperature for half an hour before the reaction was stopped by adding TCA. As might be expected, the hexose concentration in each of the three reaction mixtures fortified with TPN was

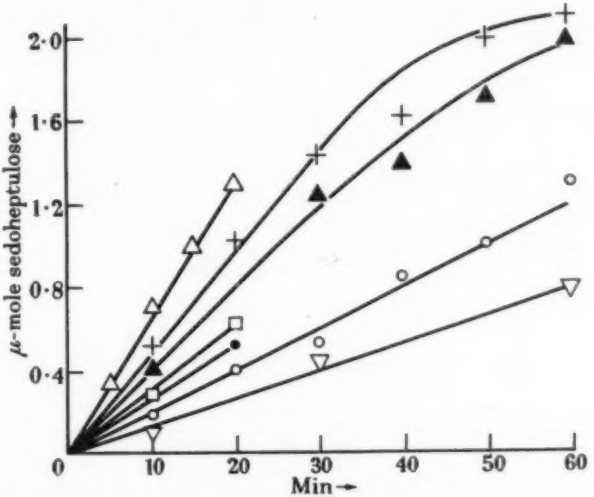

FIG. 5.—Formation of sedoheptulose from ribose 5-phosphate by soluble preparations from mouse tumours (short-term experiments).
For experimental conditions compare Fig. 2.
△——△ luteoma T 7331, +——+ lymphosarcoma T 86157, ▲——▲ *ibid.*,
□——□ mammary carcinoma T 8013, ●——● sarcoma UV 256,
○——○ *ibid.*, ▽——▽ mammary carcinoma T 98695.
Pentose breakdown at 37° C in enzyme units per gram fresh tissue amounted to 667, 615, 497, and 531 for the first four tumours respectively.

lower than their respective controls (Fig. 6). Sedoheptulose concentration did not change significantly.

(*c*) Extracts incubated with G6-P and TPN did not form sedoheptulose at 20°C, but did so at 37°C. Table III illustrates the disappearance of hexose and the formation of heptose (reactions 1–2; 3-keto 6-PG → Ru5-P + CO_2; 3–4) for a typical experiment.

The possibility that part of the sedoheptulose would be an artefact arising from the breakdown products of TPN was excluded by the finding that no ribose nor heptulose could be detected by paperchromatographic or spectrophotometric means following incubation of the tumour extracts with TPN at 37°C.

(*d*) *Reaction 8:* G6-P → F6-P (phosphohexose isomerase).

When the tumour extracts were incubated in the presence of G6-P while TPN was omitted, a rise in optical density at 420 mμ. was noted after the cysteine-sulfuric acid method was applied to aliquots of the reaction mixture at successive

times. This pointed to the formation of F6-P since the molar extinction of fructose is about twice that of glucose at this wave length. Taking this into account the concentration of glucose and fructose were calculated after it was found that no other compounds had been formed (Table IV).

(*e*) The formation of sedoheptulose could also be demonstrated by incubating the tumour extracts with G6-P and FDP. For example, 0·30 ml. of a dialysed

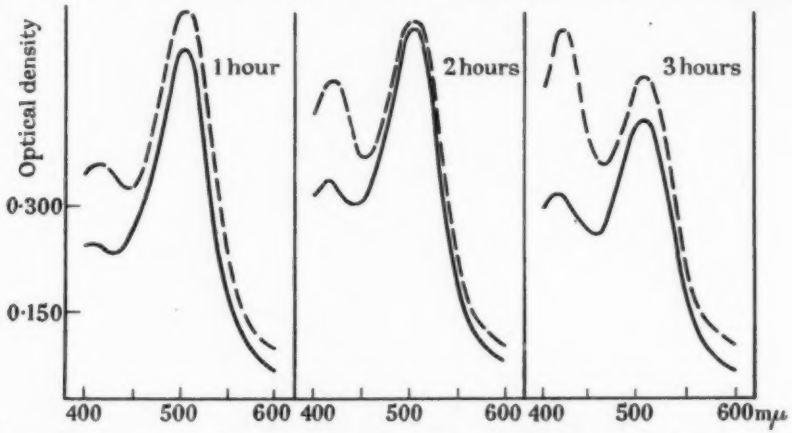

FIG. 6.—Hexose formation from ribose 5-phosphate by the soluble preparation of the ovarian tumour T 5441 both in the presence and absence of triphosphopyridine nucleotide.

The original incubation mixture consisted of 0·6 ml. 0·05 M R5-P, 1·2 ml. 0·25 M *Tris* (pH 7·6), 1·2 ml. 0·1 M $MgCl_2$ and 0·48 ml. enzyme preparation; total volume 6·0 ml. After 1, 2 and 3 hours twice 0·5 ml. was transferred and incubated with 0·1 ml. of the fresh soluble enzyme preparation and either 0·1 ml. water containing 200 μg. TPN or 0·1 ml. water alone. Incubation for 30 minutes at room temperature. After this time 4·3 ml. 5 per cent TCA was added and 1·0 ml. of the deproteinized solution was used for the cysteine-sulfuric acid test. The resulting spectra are illustrated; solid lines: from aliquots which had been incubated in the presence of TPN, broken lines: from aliquots which had been incubated in the absence of TPN.

TABLE III.—*Formation of Sedoheptulose from Glucose 6-phosphate by the Soluble Fraction of the Mouse Ovarian Tumour 5441 (Granulosa Cell Type), Incubated in the Presence of TPN.*

The reaction mixture consisted of 0·42 ml. 0·25 M *Tris*, 0·3 ml. 0·1 M $MgCl_2$, 0·15 ml. 0·05 M G6-P, 0·12 ml. enzyme preparation and 0·5 ml. 0·1 M NaOH to bring the reaction mixture, which contained also 11 mg. TPN, to a pH of 7·4. Incubation at 37°C in air. At successive times 0·2 ml. of the reaction mixture was pipetted into 1·8 ml. of 5 per cent TCA. 1·0 ml. and 0·3 ml. were used for the cysteine-sulfuric acid and the anthrone method, respectively. Simultaneously a reaction mixture containing all components except G6-P was run during the same period. Blanks containing water were treated as the test solutions.

	μ-Mole, after (minutes)				
Compound.	0.	15.	30.	60.	120.
Hexose . .	6·0	4·4	2·8	0·92	1·3
Sedoheptulose .	0	·08	·10	·38	·53

supernatant prepared from a 10 per cent homogenate of the luteoma T 7331, produced 0·70 μ-mole sedoheptulose from G6-P and FDP in 60 minutes. In this system the substrate for sedoheptulose formation is furnished by the transketolase-catalyzed reaction between F6-P and glyceraldehyde 3-P (reverse of reaction 6). F6-P is formed by reaction 8 catalyzed by phosphohexoisomerase, and glyceraldehyde 3-P by the aldolase catalyzed-reaction 7. The pentose phosphate produced will yield S7-P by reaction 3 and 4, but another route is provided by the aldolase catalyzed reaction between tetrose-P and dihydroxyacetone phosphate, recently described by Smyrniotis and Horecker (1956), in which sedoheptulose 1, 7-diphosphate is formed or by reaction 5 using tetrose-P and F 6-P.

TABLE IV.—*Formation of Fructose from Glucose 6-phosphate by the Soluble Fraction of the Mouse Sarcoma UV 256.*

This experiment was performed exactly in the same way as that illustrated in Table III, except for the fact that TPN was omitted. The conversion of glucose into fructose was calculated taking the ratio 2 : 1 in the molar extinction coefficients of fructose and glucose (cysteine-sulfuric acid method) into account.

	μ-Mole, after (minutes)			
Compound.	0.	60.	120.	150.
Glucose . .	6	2·8	3·5	2·5
Fructose . .	0	3·2	2·5	3·5

II. *Experiments with slices*

A. *Formation of sedoheptulose from endogenous sources*

First, the concentration and the rate of dissimilation of endogenous hexose was studied. Slices of the fresh tumours were cut and incubated in a Krebs Ringer phosphate buffer of pH 7·4 at 37°C with shaking in air. The two hepatomas of the mouse, T 26473 and T 28012, contained a relatively high concentration of hexose-positive reacting material in the anthrone test which fell to less than half of the original value on incubation during two hours. No convincing evidence for sedoheptulose formation was found. The hexose content of the other tumours amounted to 2–3 μ-mole per gram fresh weight at the most ; during a two hour incubation period 0–50 per cent of the hexose disappeared. A slight formation of sedoheptulose was seen after this period in slices of the ovarian tumour T 5441, the testicular tumour T 5358 and the lymphosarcoma T 86157 only. In fresh tumour tissue no heptulose could be detected by the procedures employed. The amount of heptulose produced from the endogenous substrate by the tumours just mentioned never exceeded more than ten per cent of that formed from added G6-P (compare Section II, B).

B. *Formation of sedoheptulose from added glucose and glucose 6-phosphate*

The concentration of glucose, which was added to the slices (7·5 μ-mole per 500 mg. wet weight of tissue) fell off rapidly on incubation (Table V). At no time however, did sedoheptulose appear to be present ; not even in those cases in which the heptose had been found to be formed from endogenous substrate.

TABLE V.—*Formation of Sedoheptulose from Glucose and Glucose 6-phosphate by Slices of the Sarcomatoid Ovarian Tumour T 24202.*

500 mg. slices wet weight; 7·5 μ-moles glucose or G6-P in a volume of 2·5 ml. Krebs Ringer phosphate buffer of pH 7·4. Incubation in air at 37°C. The concentrations of hexose listed in the table are those measured for the sum of endogenous and added hexose (compare text, Section II, A).

Compound added.		μ-Mole, after (minutes). 60.	120.
Glucose	Hexose	2·5	1·1
	Sedoheptulose	0	0
G6-P	Hexose	7·0	6·5
	Sedoheptulose	·45	·60

In marked contrast with the results following addition of glucose are those obtained with G6-P. Although the concentration of the latter compound fell off rather slowly, as revealed by the anthrone test, a significant rise in the optical density at 510 mμ. using the cysteine-sulfuric acid method* was found, which indicated the formation of sedoheptulose (Table V).

All the tumours which have been studied behaved exactly similarly: no sedoheptulose was formed from glucose, but the heptose was found to be present after incubation with G6-P both under aerobic and anaerobic conditions.

C. *Formation of sedoheptulose from added ribose 5-phosphate*

Incubation of slices from the ovarian tumours (sarcomatoid and granulosa cell type) with R5-P for various times yielded sedoheptulose and significant quantities of hexose (Table VI). In a few experiments in which the gas phase consisted of a 95 per cent N_2 : 5 per cent CO_2 mixture, the yield of sedoheptulose and hexose synthesized from R5-P was equal to that obtained under aerobic conditions (air). No formation of hexose could be observed when ribose was incubated with the slices.

TABLE VI.—*Formation of Sedoheptulose and Hexose from Ribose 5-phosphate by Slices of Two Ovarian Tumours (T 24202 and T 5441).*

500 mg. slices wet weight; 7·5 μ-mole R5-P in 2·5 ml. Krebs Ringer phosphate buffer of pH 7·4. Incubation in air 37° C.

Tumour.	Compound.	μ-Mole, after (minutes) 0.	30.	60.	120.	180.
T 24202	Sedoheptulose	0	—	·34	·50	—
	Hexose	1·0	—	5·0	5·0	—
T 24202	Sedoheptulose	0	·70	·68	·42	·42
T 5441	Sedoheptulose	0	—	·48	·46	—
	Hexose	1·0	—	3·8	3·7	—

* The hexose maximum at 420 mμ. may even be higher than at the start of the experiment, indicating the conversion of G6-P into F6-P.

D. *Effect of monoiodoacetate on the accumulation of intermediates*

Incubation of glucose together with MIA ($5{\cdot}4 \times 10^{-4}$ M) resulted in a marked retardation of glucose dissimilation with accumulation of F6-P (compare Section I, B, d) and of triose†, but, again, no sedoheptulose could be detected. When G6-P served as substrate, sedoheptulose formation in the presence of MIA was equal or slightly higher than in its absence.

After incubation of tumour slices with R5-P and MIA, the amount of sedoheptulose formed was not different from that found when the incubation had been carried out in the absence of MIA.

III. *Experiments with Homogenates*

Homogenates were prepared and incubated in an atmosphere of 95 per cent N_2 : 5 per cent CO_2 according to the procedures described by Novikoff, Potter and Le Page (1948) The tumours T 5441 and T 86157 were studied first. 0·3 ml. aliquots of the homogenates (10 per cent in isotonic KCl-buffer) were incubated with G6-P (15 μ-mole) in the presence of nicotinamide, fluoride, K-pyruvate and ATP, in a total volume of 3·0-ml. In the complete reaction mixture with DPN and TPN present, sedoheptulose was formed. When DPN, or DPN and TPN, were omitted the formation of sedoheptulose was not diminished. On the contrary, under the latter conditions the greatest amount of sedoheptulose was found, while the hexose disappearance was minimal as compared with the other experiments (Table VII).

TABLE VII.—*Formation of Sedoheptulose from Glucose 6-phosphate by a Fortified Homogenate of the Lymphosarcoma T 86157.*

The reaction mixture consisted of 0·3 ml. 0·1 M $(NH_4)_3PO_4$, 0·3 ml. 0·5 M KCl in 0·5 M $KHCO_3$, 0·2 ml. 0·01 M K-ATP, 0·3 ml. 0·4 M nicotinamide, 0·1 ml. 0·1 M $MgCl_2$, 0·1 ml. 0·15 M K-pyruvate, 0·19 ml. 0·16 M NaF, 0·3 ml. 0·05 M G6-P.

0·3 ml. 10 per cent tumour homogenate in isotonic KCl (containing bicarbonate) (final pH 7·7–8·1). Added 1·5 mg. TPN plus 0·5 mg. DPN, 1·0 mg. DPN, 1·5 mg. TPN or water only. Total volume 3·0 ml. Incubation at 37° C in an atmosphere of 95 cent N_2 plus 5 per cent CO_2 for 1 or 2 hours.

Coenzymes added.	Hexose disappeared after (minutes). 60. (μ-mole.)	120. (μ-mole.)	Sedoheptulose formed after (minutes). 60. (μ-mole.)	120. (μ-mole.)
DPN + TPN	11·1	11·3	·28	·25
DPN	9·6	9·4	·44	·39
TPN	6·8	9·4	·54	·51
None	3·0	3·0	·81	·90

In the absence of DPN, TPN, ATP, nicotinamide (the inhibitor of the pyridine nucleotide-destroying enzymes), fluoride (the inhibitor of the " ATP-splitting

† The formation of triose was evident from the rise in optical density at 450 mμ (anthrone test). In the following experiments using MIA together with G6-P or R5-P, triose was also found to accumulate.

enzyme ") and pyruvate, sedoheptulose was still formed. For example, in an experiment with T 5441 0·65 and 0·98 μ-mole sedoheptulose were found after 1 and 2 hours, respectively. Similar results were obtained with a number of other tumours.

In a number of experiments attempts were made to destroy the TPN, ATP and DPN which might still be present in the homogenate. This was accomplished by pre-incubation of the latter for 15 minutes at 37°C in the presence of sucrose mitochondria prepared according to Schneider and Hogeboom (1950) from a transplanted liver carcinoma of the rat or from the sarcomatoid ovarian tumour T 24202. The mitochondria from these tumours had been found to possess high TPN-, DPN- and ATP-splitting activities. (Emmelot, Bos and Brombacher, 1956).

To serve this purpose the mitochondria were freed from sucrose by washing with and dialysis against distilled water. Despite the pre-treatment of the homogenate with the mitochondria, sedoheptulose formation by the unfortified homogenate as described for the previous experiment, remained unchanged; hexose concentration did not fall.

In a separate experiment with the mitochondria incubated without the homogenate no heptose was found.

DISCUSSION

The experiments with slices reported in the present communication have shown that sedoheptulose, an important intermediate of the extra-glycolytic pathway, could not be demonstrated when glucose was used as the substrate. Thus sedoheptulose did not accumulate although it was undoubtedly formed. However with G6–P, which is markedly slower metabolized than free glucose, probably as a result of a more rapid entrance of the latter into the cells, sedoheptulose was found to accumulate in the tumour slices. Sedoheptulose could also be demonstrated when fortified whole homogenates of the tumours were incubated with G6-P. Sedoheptulose formation was at its maximum and hexose disappearance markedly less when the two coenzymes of the glycolytic and extra-glycolytic pathway, DPN and TPN, were omitted from the incubation mixture. After pre-treatment of the homogenates with TPN-, DPN- and ATP-splitting enzymes from mitochondrial preparations of certain tumours, and subsequent incubation of the homogenate sedoheptulose was still formed in the absence of added coenzymes, inhibitors (fluoride and nicotinamide), pyruvate, and ATP. It is improbable that under the latter conditions heptose originated from ribose produced by the direct oxidative decarboxylation of G6-P. Sedoheptulose formation by the pathway described under Section I, B, e, *i.e.* from F6-P and T3-P, would be more plausible. The former compound can easily be formed from added G6-P, whereas the latter or its direct precursor FDP has to be present in the original homogenate to account for this reaction.

It appeared further, that sedoheptulose accumulation was favoured under conditions in which glycolysis was abolished or retarded. A similar inverse relation between substrate metabolism via the glycolytic path and sedoheptulose accumulation was found in the slice experiments using glucose and G6-P. However, in the presence of MIA, which blocked the glycolytic pathway, glucose did not give rise to sedoheptulose.

The experiments carried out with cell-free extracts unambiguously demonstrated that the enzymes of the HMP-oxidative pathway were present in all the tumours studied. Apart from those described, still other reactions may be involved. Phosphorylation of Ru5-P and a carboxylation of the ribulose diphosphate formed with subsequent fission of the resulting six carbon compound into two molecules phosphoglyceric acid was postulated by Bassham, Benson and Calvin (1950) to account for the early appearance of the latter compound during their photosynthetic studies. The pertinent reactions have recently been demonstrated by Weissbach, Horecker and Hurwitz (1956) and by Jakoby, Brummond and Ochoa (1956). In accordance with this reaction sequence Barron and co-workers (1955) have found ^{14}C exclusively located in the carboxyl group of the lactic acid which was isolated after anaerobic incubation of tissue slices with $H^{14}CO_3^-$. Apparently these reactions were also involved in the pentose metabolism of the tumours studied by us. It was found (van Vals, Bosch and Emmelot, unpublished) that the lactic acid isolated after aerobic incubation of glucose-1-^{14}C with slices from the transplanted mammary carcinoma T 49985, the lymphosarcoma T 86157 and the sarcomatoid ovarian tumour T 24202, contained three to five times as much isotope in its carboxyl group than did the corresponding group of the lactic acid originating from glucose-6-^{14}C. Both carbon atoms 1 and 6 of glucose are converted to the methyl group of lactate, but since $^{14}CO_2$ with a higher specific activity is released from glucose-1-^{14}C than from glucose-6-^{14}C during incubation of the tumour slices (van Vals, Bosch and Emmelot, 1956) the pentose carboxylation reaction may account for our findings.

SUMMARY

1. By the use of cell-free extracts, homogenates and slices of transplanted tumours, enzymes, intermediates and reactions participating in the hexose monophosphate oxidative pathway were demonstrated.

2. In the cell-free extracts, dehydrogenation involving glucose 6-phosphate and 6-phosphogluconate, ribolysis and formation of sedoheptulose, hexose, and triose were studied. All enzymes involved in the HMP oxidative pathway were found present.

3. Slices incubated in the presence of glucose 6-phosphate produced sedoheptulose. When added glucose was the substrate, sedoheptulose did not accumulate. On incubation with ribose 5-phosphate, sedoheptulose and hexose were produced.

4. In fortified and unfortified whole homogenates, sedoheptulose was formed from glucose 6-phosphate.

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INDEX TO AUTHORS

ADAMS, D. H., sex differences in tissue catalase levels, and their relation to the catalase-depressing action of tumours, 748

AIRD, I., WEINBREN, K. and WALTER, L., angiosarcoma in a limb the seat of spontaneous lymphoedema, 424

ALVORD, E. T. and CARDON, S. Z., the inhibition of formation of 3,4–benzpyrene in cigarette smoke, 498

——— see *Cardon, S. Z.*

ANDERSON, A. J., see *Lockey, Eunice.*

ARGUS, MARY F., HEWSON, KATHLEEN and RAY, FRANCIS E., effect of tumors on liver and spleen uptake of radioactive material, 321

ATKIN, N. B. and RICHARDS, B. M., deoxyribonucleic acid in human tumours as measured by microspectrophotometry of Feulgen stain: a comparison of tumours arising at different sites, 769

BADGER, G. M., miscellaneous chemical carcinogens: chemical constitution and carcinogenic activity, 330

BARNES, J. M., see *Magee, P. N.*

BERENBLUM, I., see *Haran, Nechama.*

BIELSCHOWSKY, F., see *Bielschowsky, Marianne.*

BIELSCHOWSKY, MARIANNE, BIELSCHOWSKY, F. and LINDSAY, DIANA, a new strain of mice with a high incidence of mammary cancers and enlargement of the pituitary, 688

BONSER, GEORGIANA M., BRADSHAW, L., CLAYSON, D. B. and JULL, J. W., a further study of the carcinogenic properties of ortho hydroxy-amines and related compounds by bladder implantation in the mouse, 539

——— CLAYSON, D. B. and JULL, J. W., the induction of tumours of the subcutaneous tissues, liver and intestine in the mouse by certain dyestuffs and their intermediates, 653

——— ——— ——— and PYRAH, L. N., the carcinogenic activity of 2-naphthylamine, 533

BOS, C. J., see *Emmelot, P.*

BOSCH, L., VAN VALS, G. H. and EMMELOT, P., the metabolism of neoplastic tissues: demonstration of intermediates and reaction sequences of the hexose monophosphate oxidative pathway, 801.

BOSCH, L., see *van Vals, G. H.*

BRADSHAW, L., see *Bonser, Georgiana M.*

BROMBACHER, P. J., see *Emmelot, P.*

BROMBERG, Y. M. and ERDREICH, M. B., changes in volume of normal erythrocytes incubated with plasma of patients with malignant tumours, 763

BUTLER, J. A. V., JOHNS, E. W., LUCY, J. A. and SIMSON, P., the composition of nucleic acids prepared from rat and mouse tumours, 202

CALCUTT, G., the intracellular metabolism of 3 : 4 benzpyrene. The role of enzymes in the formation of benzpyrene metabolites by liver components, 583

CAMPBELL, J. G., two cases of rhabdomyosarcoma in the fowl, 700

CAMPBELL, J. M. and KREYBERG, L., the degree of air pollution in Norwegian towns, 481

——— and LINDSEY, A. J., polycyclic hydrocarbons extracted from tobacco; the effect upon total quantities found in smoking, 649

CARR, J. G., renal adenocarcinoma induced by fowl leukaemia virus, 379

CARDON, S. Z., ALVORD, E. T., RAND, H. J. and HITCHCOCK, R., 3,4–benzpyrene in the smoke of cigarette paper, tobacco and cigarettes, 485

——— see *Alvord, E. T.*

CHALMERS, J. G., separation of 3,4-benzpyrene metabolites by paper partition chromatography, 787

——— and PULLINGER, B. D., test of some steroid substances for carcinogenic action in mouse bladders, 547

CHESTERMAN, F. C., see *Franks, L. M.*

CLAYSON, D. B., see *Bonser, Georgiana M.*

COHEN, A. and COHEN, L., radiobiology of the C3H mouse mammary carcinoma: increased radiosensitivity of the tumour induced by inoculation of the host with radiation-attenuated isografts, 312

COHEN, L., see *Cohen, A.*

COMMINS, B. T. and LINDSEY, A. J., some phenolic constituents of cigarette smoke, 504

CRABTREE, H. G., the effects of sulphonamido-azo-compounds on azo-carcinogenesis, 129

CUDKOWICZ, G., the polysaccharides of a human carcinoma, 758

DAVIS, R. A., see *Rook, A. J.*
DELORY, G. E., see *Israels, L. G.*

EDINGTON, G. M., malignant disease in the Gold Coast, 595
ELKELES, A., cancer and atherosclerosis, 247
EMMELOT, P., BOS, C. J., and BROMBACHER, P. J., fatty acid oxidation in normal and neoplastic tissues, 188
——— see *Bosch, L.*
——— see *van Vals, G. H.*
EPSTEIN, M. A., the identification of the Rous virus; a morphological and biological study, 33
ERDREICH, M. B., see *Bromberg, Y. M.*

FRANKS, L. M. and CHESTERMAN, F. C., intra-epithelial carcinoma of prostatic urethra, peri-urethral glands and prostatic ducts ("Bowen's disease of urinary epithelium"), 223

GALLUZZI, S. and PAYNE, P. M., brain metastases from primary bronchial carcinoma: a statistical study of 741 necropsies, 408
GHADIALLY, F. N. and WISEMAN, G., the effect of excess dietary methionine on the rate of growth of RD3 sarcoma, 570
GILBERT, J. A. S. and LINDSEY, A. J., polycyclic hydrocarbons in cigarette smoke: the amounts held in stubs and ash, 642
——— ——— polycyclic hydrocarbons in tobacco smoke: pipe smoking experiments, 646
GILLMAN, T., HATHORN, M. and PENN, J., apparent paradoxical effects of lanolin on induction of skin and lung tumours by topically applied methylcholanthrene. A preliminary report, 384
——— ——— ——— actions of cortisone on cutaneous and pulmonary neoplasms induced in mice by cutaneous applications of methylcholanthrene, 394
GLENDENNING, O. M., see *Roe, F. J. C.*
GRENER, CAROL, see *Wynder, E. L.*
GRESHAM, G. A., see *Rook, A. J.*
GUIMARAES, J. P. and LAMERTON, L. F., further experimental observations on the late effects of thorotrast administration, 527
GUTHRIE, J., attempts to produce seminomata in the albino rat by inoculation of hydrocarbons and other carcinogens into normally situated and ectopic testes, 134

HADFIELD, GEOFFREY and YOUNG, J. STRETTON, the mammotrophic potency of human urine, 145
——— ——— the mammotrophic potency of the urine of normal post-menopausal women, 324
HAMER, D. and WOODHOUSE, D. L., biological tests for carcinogenic action of tar from cigarette smoke, 49
HARAN, NECHAMA and BERENBLUM, I., the induction of the initiating phase of skin carcinogenesis in the mouse by oral administration of urethane (ethyl carbamate), 57
HATHORN, M., see *Gillman, T.*
HEATH, J. C., the production of malignant tumours by cobalt in the rat, 668
HEWITT, H. B., the quantitative transplantation of Sarcoma 37 into subcutaneous air pouches in mice, 564
HEWSON, KATHLEEN, see *Argus, Mary F.*
HIGGINSON, J., primary carcinoma of the liver in Africa, 609
HITCHCOCK, R., see *Cardon, S. Z.*
HORNING, E. S., observations on hormone-dependent renal tumours in the golden hamster, 678

ISRAELS, L. G., and DELORY, G. E., serum isomerase in leukemia, 318
IVERSEN, HANS-GEORG, human tumours in cortisone-treated mice, 472

JELLIFFE, A. M. and MACIVER, J. E., desacetylmethylcolchicine in the treatment of myeloid leukaemia, 634
JOHNS, E. W., see *Butler, J. A. V.*
JULL, J. W., see *Bonser, Georgiana M.*

KOLLER, P. C. and VERONESI, U., the effects of a nitrogen mustard derivative of phenylalanine on experimental tumours, 703
KORTEWEG, R., the significance of selection in prospective investigations into an association between smoking and lung cancer, 282
KREYBERG, L., see *Campbell, J. M.*

LAMERTON, L. F., see *Guimaraes, J. P.*
LAPIS, K. and NÉMETH, L., effect of various chemotherapeutic agents on metastasis, 719
LARIONOV, L. F., some biological and clinical results from the investigations of the chloroethylamines as anti-tumour drugs, 26
LASNITZKI, ILSE, the effect of 3–4-benzpyrene on human foetal lung grown *in vitro*, 510

LINDSAY, DIANA, see *Bielschowsky, Marianne.*
LINDSEY, A. J., see *Campbell, J. M.*
——— see *Commins, B. T.*
——— see *Gilbert, J. A. S.*
LIPSCHUTZ, A., see *Mardones, Elvira.*
LOCKEY, EUNICE, ANDERSON, A. J. and MACLAGAN, N. F., urine and serum mucoproteins in cancer and other diseases, 209
LUCAS, R. B., see *Ranger, D.*
LUCY, J. A., see *Butler, J. A. V.*
LUPBERGER, A., see *Wynder, E. L.*

MACIVER, J. E., see *Jelliffe, A. M.*
MCKENZIE, A., duration of symptoms, clinical staging and survival in cancer of certain sites, 401
MCKEOWN, FLORENCE, malignant disease in old age, 251
MACLAGAN, N. F., see *Lockey, Eunice.*
MAGEE, P. N. and BARNES, J. M., the production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine, 114
MARDONES, ELVIRA and LIPSCHUTZ, A., on the influence of cortisone on the evolution of tumoural growth in intrasplenic ovarian grafts in two strains of mice, 517
MARKS, JOHN, see *Pillers, E. M. K.*
MARRIAN, D. H. and MAXWELL, D. R., tracer studies of potential radiosensitizing agents. Tetrasodium 2-^{14}C-methyl-1 : 4-naphthohydroquinone diphosphate, 575
——— ——— tracer studies of potential radiosensitizing agents. Tetrasodium 2 - methyl - 3 - ^{82}Br - bromo - 1 : 4 - naphthohydroquinone diphosphate and tetrasodium 2 : 3-dimethyl-5 : 6-di ^{131}I - iodo - 1 : 4 - benzohydroquinone diphosphate, 739
MARSHALL, A. H. E., reactions in lymph nodes draining X-irradiated and carcinogen treated tissues, 307
MAXWELL, D. R., see *Marrian, D. H.*
METCALF, D., a lymphocytosis stimulating factor in the plasma of chronic lymphatic leukaemic patients, 169
——— further studies on the plasma lymphocytosis stimulating factor in chronic lymphatic leukaemia and some other disease states, 431
——— the thymic origin of the plasma lymphocytosis stimulating factor, 442
MILLER, E. W. and PYBUS, F. C., hyperplastic and neoplastic lesions of the glandular stomach and intestine in two inbred strains of mice and their reciprocal hybrids, 89
MITCHELL, J. S., see *Pillers, E. M. K.*
NEISH, W. J. P., polarimetric investigation of sera from normal and cancer rats, 179
NÉMETH, L., see *Lapis, K.*

O'CONNOR, R. J., the proliferative capacity of the lining epithelium of the anal canal in mice, 733
O'NEAL, MARGERY A., see *Reid, E.*

PAXON, T. [illegible] a study of tuberculosis and cancer [illegible]ality rates with special reference to lung cancer rates, 623
PAYNE, P. M., see *Galluzzi, S.*
PEACOCK, ANDRÉE, attempts at oral transmission of mammary tumour agent by feeding male organs, 715
——— and PEACOCK, P. R., methylcholanthrene-induced tumours of glandular epithelium in fowls, 110
PEACOCK, P. R., see *Peacock, Andrée.*
PEARSON, A. E. G., the effects of fractionated X-irradiation on the growth of Sarcoma 37 in mice, 674
PENN, J., see *Gillman, T.*
PETERSEN, M., see *Torgersen, O.*
PHILPS, F. R., vacuolated cells in the sputum simulating adenocarcinoma cells, 24
PILLERS, E. M. K., MARKS, JOHN and MITCHELL, J. S., the bone marrow in malignant disease, 458
PULLINGER, B. D., see *Chalmers, J. G.*
PYBUS, F. C., see *Miller, E. W.*
PYRAH, L. N., see *Bonser, Georgiana M.*

RAND, H. J., see *Cardon, S. Z.*
RANGER, D., THACKRAY, A. C. and LUCAS, R. B., mucous gland tumours, 1
RAY, FRANCIS, E., see *Argus, Mary F.*
REID, E., carcinogenic action of 2′-chloro-4-dimethylaminostilbene, in relation to (A) possible effect of growth hormone, and (B) composition of liver cytoplasm, 123
——— and O'NEAL, MARGERY, A., "free" enzymic activity of mitochondrial fractions from liver tumours, 587
RICHARDS, B. M., see *Atkins, N. B.*
RICHARDSON, W. W., medullary carcinoma of the breast, 415
ROE, F. J. C., the development of malignant tumours of mouse skin after "initiating" and "promoting" stimuli. I. The effect of a single application of 9,10-dimethyl-1, 2-benzanthracene (DMBA), with and without subsequent treatment with croton oil, 61

ROE, F. J. C., the development of malignant tumours of mouse skin after "initiating" and "promoting" stimuli. III. The carcinogenic action of croton oil, 72
——— and GLENDENNING, O. M., the carcinogenicity of β-propiolactone for mouse-skin, 357
——— see *Salaman, M. H.*
ROOK, A. J., GRESHAM, G. A. and DAVIS, R. A., squamous epithelioma possibly induced by the therapeutic application of tar, 17

SAFFIOTTI, U. and SHUBIK, P., the role of burning in carcinogenesis, 54
SALAMAN, M. H. and ROE, F. J. C., the development of malignant tumours of mouse skin after "initiating" and "promoting" stimuli. II. The influence of alternate applications of croton oil on malignant tumour- production by repeated applications of dilute 9,10-dimethyl-1,2-benzanthracene (DMBA), 70
——— ——— the development of malignant tumours of mouse skin after "initiating" and "promoting" stimuli. IV. Comparison of the effects of single and divided initiating doses of 9,10 - dimethyl - 1,2 - benzanthrene (DMBA), 79
——— ——— further tests for tumour-initiating activity: N,N-di-(2-chloroethyl) - *p* - aminophenylbutyric acid (CB1348) as an initiator of skin tumour formation in the mouse, 363
SANDERUD, K., squamous epithelial metaplasia in the respiratory tract in uraemics. A preliminary report on a study of post mortem cases, 226
SHANMUGARATNAM, K., primary carcinomas of the liver and biliary tract, 232
SHUBIK, P., see *Saffiotti, U.*
SIMSON, P., see *Butler, J. A. V.*
STOCKS, P., cancer death rates in Japan contrasted with those in England and Wales and Canada, 257

THACKRAY, A. C., see *Ranger, D.*
TORGERSEN, O. and PETERSEN, M., the epidemiology of gastric cancer in Oslo: material, diagnosis and mobility of population. Some sources of error, 292
——— ——— the epidemiology of gastric cancer in Oslo: cartographic analysis of census tracts and mortality rates of sub-standard housing areas, 299
TROMP, S. W., the geographical distribution of cancer of the stomach in the Netherlands (period 1946–1952), 265

VAN VALS, G. H., see *Bosch, L.*
———, BOSCH, L. and EMMELOT, P., the metabolism of neoplastic tissues: carbon dioxide production from specifically ^{14}C-labelled glucose by normal and neoplastic tissues, 792
VERONESI, U., see *Koller, P. C.*

WALLACE, A. CAMERON, the occurrence of metastases in a group of related rat tumours, 724
WALTER, L., see *Aird, I.*
WEILER, E., antigenic differences between normal hamster kidney and stilboestrol induced kidney carcinoma: complement fixation reactions with cytoplasmic particles, 553
——— antigenic differences between normal hamster kidney and stilboestrol induced kidney carcinoma: histological demonstration by means of fluorescing antibodies, 560
WEINBREN, K., see *Aird, I.*
WISEMAN, G., see *Ghadially, F. N.*
WOODHOUSE, D. L., see *Hamer, D.*
WYNDER, E. L., LUPBERGER, A. and GRENER, CAROL, experimental production of cancer with cigarette tar: strain differences, 507

YOUNG, J. STRETTON, see *Hadfield, Geoffrey.*